DEVELOPMENT OF MICROPARTICLES FOR ORAL VACCINE DELIVERY

By
Miss Tittaya Suksamran

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy Program in Pharmaceutical Technology
Graduate School, Silpakorn University
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การพัฒนาระบบไมโครพาร์ทิเคิลสำหรับการนำส่งวัคซีนทางการรับประทาน

โดย
นางสาวทิษฎยา สุขสำราญ

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The purpose of this study was to prepare the microparticles entrapping ovalbumin (OVA) as the model antigen in order to induce immune responses in mice following oral vaccination. In this study, calcium-alginate (Ca-alginate) and calcium-yam-alginate (Ca-Yam-alginate) microparticles were prepared by cross-linking alginate with calcium chloride solution ($CaCl_2$) using an electrohydrodynamic spraying technique. The effects of alginate and calcium chloride concentration and electrical potential on particle size and shape, OVA entrapment efficiency and content were investigated. The results showed that 1 %w/v alginate low viscosity (ALV), 4 %w/v $CaCl_2$, electrical potential 20 kV was the appropriate condition for OVA incorporation. Four chitosan derivatives namely trimethylated chitosan (TM), methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (Bz), methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan (CM) and methylated *N*-(4-pyridinylmethyl) chitosan (Py) were investigated for their cytotoxicity and immunological activity. CM showed higher IgG and IgA titres than TM (16 times), Bz (10 times) and Py (7 times) by oral administration. Therefore, CM was selected for coating on Ca-alginate and Ca-Yam-alginate microparticles entrapping initial OVA of 10, 20 and 40% w/w to polymer. The optimized concentration of CM for complete coating on microparticles was 0.1 %w/v. The coated microparticles (CM-Ca-alginate and CM-Ca-Yam-alginate microparticles) was spherical and had smooth surface with the average size of 1-3 µm and positively charges. In addition, they had higher degree of swelling and mucoadhesive properties than uncoated microparticles. The result of OVA content and entrapment efficiency revealed that at initial, 40% OVA yielded the highest entrapment efficiency and OVA content. The entrapment efficiency and OVA content of coated microparticles were lower than uncoated microparticles. The *in vitro* release of microparticles showed that coated microparticles resulted in sustained release more than uncoated microparticles. Cytotoxicity results showed that all formulations were safe. The *in vivo* oral administration demonstrated that at the same amount of 250 µg OVA, CM coated microparticles exhibited the highest *in vivo* adjuvant activity in both IgG and IgA immunogenicity.

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วัตถุประสงค์งานวิจัยนี้เพื่อเตรียมอนุภาคไมโครพาร์ทิเคิลที่กักเก็บแอนติเจนต้นแบบโอวัลบูมิน เพื่อหวังผลในการเหนี่ยวนำให้เกิดการตอบสนองทางภูมิคุ้มกันในหนูทดลองทางการรับประทาน ในการศึกษาครั้งนี้แคลเซียมอัลจิเนตและแคลเซียมแยมอัลจิเนตไมโครพาร์ทิเคิลเตรียมขึ้นจากกระบวนการเชื่อมพันธะระหว่างอัลจิเนตและแคลเซียมคลอไรด์โดยใช้เทคนิคกระบวนการพ่นทางไฟฟ้าสถิตย์ จากนั้นทำการศึกษาผลของความเข้มข้นของอัลจิเนตและแคลเซียมคลอไรด์ และกระแสไฟฟ้าที่ให้ต่อลักษณะและขนาดของอนุภาคที่เกิดขึ้น อีกทั้งยังศึกษาเปอร์เซ็นต์ในการกักเก็บและปริมาณการกักเก็บโอวัลบูมินภายในอนุภาค พบว่าอัลจิเนตความหนืดต่ำและแคลเซียมคลอไรด์ที่ความเข้มข้น 1 และ 4 เปอร์เซ็นต์ต่อปริมาตร กระแสไฟฟ้า 20 กิโลโวลต์ เป็นสภาวะที่เหมาะสมสำหรับนำมากักเก็บโอวัลบูมิน ต่อมานำอนุพันธุ์ไคโตซานทั้งสี่ชนิดได้แก่ trimethylated chitosan (TM), methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (Bz), methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan (CM) and methylated *N*-(4-pyridinylmethyl) chitosan (Py) มาศึกษาความเป็นพิษและฤทธิ์ในการกระตุ้นภูมิคุ้มกันในหนูทดลองทางการรับประทาน พบว่า CM ให้ผลในการกระตุ้นภูมิคุ้มกันในกระแสเลือดและระบบเยื่อเมือกสูงกว่า TM (16 เท่า) Bz (10 เท่า) และ Py (7 เท่า) ตามลำดับ ดังนั้นจึงเลือก CM มาเคลือบบนแคลเซียมอัลจิเนตและแคลเซียมแยมอัลจิเนตไมโครพาร์ทิเคิลที่กักเก็บโอวัลบูมินไว้ปริมาณ 10, 20 และ 40 เปอร์เซ็นต์ต่อน้ำหนักพอลิเมอร์ ความเข้มข้นที่เหมาะสมของ CM ที่สามารถเคลือบบนพื้นผิวของไมโครพาร์ทิเคิลได้อย่างสมบูรณ์ได้แก่ 0.1 เปอร์เซ็นต์ต่อปริมาตร ลักษณะทางกายภาพของไมโครพาร์ทิเคิลที่เคลือบด้วย CM มีรูปร่างกลม พื้นผิวเรียบโดยมีขนาดอยู่ในช่วง 1-3 ไมโครเมตร และมีประจุบนพื้นผิวเป็นบวก ด้านคุณสมบัติการพองตัวและการเกาะติดเยื่อเมือกของไมโครพาร์ทิเคิล พบว่าไมโครพาร์ทิเคิลที่เคลือบด้วย CM จะมีคุณสมบัติในการพองตัวและการเกาะติดเยื่อเมือกสูงกว่าไมโครพาร์ทิเคิลที่ไม่ได้เคลือบ ผลการกักเก็บโอวัลบูมินภายในไมโครพาร์ทิเคิลพบว่าที่โอวัลบูมินปริมาณ 40 เปอร์เซ็นต์ต่อน้ำหนักพอลิเมอร์ให้เปอร์เซ็นต์และปริมาณการกักเก็บโอวัลบูมินสูงที่สุด และพบว่าไมโครพาร์ทิเคิลที่เคลือบด้วย CM จะให้เปอร์เซ็นต์ในการกักเก็บและปริมาณในการกักเก็บที่ต่ำกว่าไมโครพาร์ทิเคิลที่ไม่ได้เคลือบ การทดสอบการปลดปล่อยโอวัลบูมินออกจากไมโครพาร์ทิเคิลภายนอกร่างกายพบว่าไมโครพาร์ทิเคิลที่เคลือบด้วย CM จะให้ผลการปลดปล่อยที่ช้ากว่าไมโครพาร์ทิเคิลที่ไม่ได้เคลือบ ผลการทดสอบความเป็นพิษพบว่าอนุภาคไมโครพาร์ทิเคิลทุกรูปแบบมีความปลอดภัยในการนำมาใช้ โดยผลการทดสอบการกระตุ้นภูมิคุ้มกันในหนูทดลองเมื่อนำส่งไมโครพาร์ทิเคิลทางการรับประทานพบว่า ที่ปริมาณโอวัลบูมิน 250 ไมโครกรัม ไมโครพาร์ทิเคิลที่เคลือบด้วย CM สามารถกระตุ้นภูมิคุ้มกันทั้งอิมมูโนโกลบูลินชนิดG และอิมมูโนโกลบูลินชนิดAในหนูทดลองสูงที่สุด

ภาควิชาเทคโนโลยีเภสัชกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ลายมือชื่อนักศึกษา..... ปีการศึกษา 2554
ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. …….. 2. ..……..……... 3. ….……

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the research problem

Vaccine is a material that induces an immunologically mediated resistance to a disease but not necessarily an infection. Vaccines are generally composed of killed or attenuated organisms or subunits of organisms or DNA encoding antigenic proteins of pathogens. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters (Saroja, 2011: 65-74). Most of vaccinations are performed by parenteral administration. Parenteral vaccinations require trained personnel and sterilized materials, and infusion often causes noncompliance to patients (Mika, 2005: 223-234). Moreover, most exogenous pathogenic infections enter their host by the mucosal route. Thus, stimulation of mucosal defenses is very important for controlling infection and preventing disease. Induction of local responses is possible through the use of mucosal vaccines as parenteral vaccination elicits little or no IgA, the antibody isotype that is abundantly secreted in mucosal tissues and that is responsible for their defense (McGhee, 1992: 75-88). Mucosal vaccinations include buccal, intranasal, oral etc. Among these vaccinations, oral is a highly attractive approach due to its convenience for both patient and practitioners and low costs. However, development of a potent oral vaccine carrier is still hindered due to degradation of the antigen in the gastrointestinal (GI) tract and inefficient targeting to the site of action in the gut. Before the antigen reaches the M-cells of the Peyer's patches in the GI tract, it has to pass the stomach with its low pH and several inactivating enzymes. Even if the vaccine has arrived near the Peyer's patches, an immune response is not always elicited. The antigen might not reach the dome of the Peyer's patch due to inefficient uptake, or is not potent enough to provoke a sufficient immune reaction (Aizpurua, 1988: 440). In order to achieve the successful oral delivery of vaccines, they need to be protected from the harsh environment in the

stomach. One approach to enhance the bioavailability of vaccines, currently the subject of intense research, is the use of microparticles as drug/vaccine carriers. Association with an appropriate microparticulate carrier should confer numerous advantages upon a poor bioavailability vaccine, including protection from degradation (enzymatic and environmental), reduction of nonspecific interactions with food proteins and facilitation of uptake by the gut-associated lymphoid tissue (GALT) (Anya, 1998: 69-75).

Microparticles are particles ranging in size between 1-1000 μm. They have a drug entrapped, adsorbed or dispersed within particles. Microparticles can be divided into 2 types, namely microcapsule and microsphere. Microcapsule is composed of a polymeric wall containing a liquid inner core where the drug is entrapped, while microsphere is made of a solid polymeric matrix in which the drug can be dispersed. Active substances may be either adsorbed at the surface of the polymer or encapsulated within the particles. Particles may be produced by polymerization of synthetic monomers, or dispersion of synthetic polymers or natural macromolecules (Elzoghby, 2011: 206-16). Microparticles delivery systems are extensively investigated as a drug delivery strategy in the pharmaceutical research. In general, microcarriers may protect a drug from degradation, enhance drug absorption by facilitating diffusion through epithelium, modify pharmacokinetic and drug tissue distribution profile and/or improve intracellular penetration and distribution (Florence, 2005: 65-80). Recently, they have many studies in polymeric microparticles for oral vaccine delivery, for example, Lubben and co-workers prepared chitosan microparticles and characterized with respect to size, zeta potential, morphology and ovalbumin loading and release. Furthermore, the in vivo uptake of chitosan microparticles by murine Peyer's patches was studied using confocal laser scanning microscopy (CLSM). The results showed that chitosan microparticles which smaller than 10 μm can be taken up by M-cells of Peyer's patches. As such they are suitable to serve as vaccination systems (Lubben, 2001: 687-694). Rydell et al. investigated biodegradable starch microparticles as a carrier in a mucosal vaccine against diphtheria. The result showed a strong systemic immune response after oral immunization. The method has been used for the formulation of an oral candidate vaccine to be trialed in healthy volunteers (Rydell, 2005: 2775–2783). The use of

microparticles as vaccine delivery tools poses a number of questions regarding the specific role of the particle in vaccination and stimulation of an immune response. One central question, 'Do particles act as adjuvants?' is in fact a very complex one. An immunologic adjuvant is defined as any substance that acts to accelerate, prolong, or enhance antigen specific immune responses (Sasaki, 2000: 241-249).

For designing oral dosage forms, the formulator must consider that the natural pH environment of GI tract varies from acidic (pH 1.2) in the stomach to slightly alkaline in the intestine (pH 7.4) (Shargel, 1999). In the design of oral delivery of peptide or protein drugs, pH sensitive hydrogels have increasingly attracted. Swelling of such hydrogels in the stomach should be minimal and thus the releasing of drug will be reduced as well. The extent of swelling increases as the hydrogels pass down the intestinal tract due to an increase in pH. A variety of synthetic or natural polymers with acidic or basic pendant groups have been employed to fabricate pH sensitive hydrogels (Amani, 2005: 94-101). The main criteria dictating polymer eligibility for drug delivery have been bioavailability, biocompatibility, straight forward production and degradation rate, which provide a sustained release of drugs encapsulated in microparticles. Starch, alginate, and chitosan are naturally occurring biopolymers that are finding widespread applications in food and pharmaceutical industry.

Starch is a carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. Starch is one of the most important natural organic compounds, abundant in nature. This polysaccharide is produced by all green plants as an energy store. It is found in the roots or fruits. The most common sources of food starch are corn, potato, wheat, tapioca and rice. Over time, demand for starch by non-food industries will tend to grow faster than demand for starch by food industries. In recent years, pharmaceutical companies widely use starches in various stages of drug development technology. Excipient plays a very important role in solid dosage formulation by imparting mechanical strength, stability and tablet disintegration.

Alginate is used extensively in food industry as a thickener, an emulsifier and a stabilizer. The hydrogel properties of calcium alginate beads have been proposed for controlling the release of small molecules and macromolecules

(Gombotz, 1995: 267–285). In addition, being mucoadhesive, these beads are likely to stick to the intestinal mucosa for a prolonged period of time and have been exploited for the site-specific drug delivery to mucosal tissues (Chickering, 1997: 35–46). Alginate has been extensively utilized for oral immunization of humans and animals by targeting to the Peyer's patches in small intestine (Rastogi, 2007: 71-77). Alginate can encapsulate drugs or biological molecules such as insulin, lipophilic drugs or large biomolecules by using various techniques like emulsification, emulsification/internal gelation and water/oil/water emulsification to produce microspheres (Susana, 2007: 725–731).

Chitosan (CS) is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity (Muzzarelli, 1997: 131 –140). It is soluble only in acidic milieu, in which the amino groups at the C-2 position of CS are protonated resulting in positively charges (Illum, 2001: 81– 96). These positive charges contribute to the mucoadhesive property of CS by forming strong electrostatic interaction with the negatively charged sialic acid residues of mucins resulting in an increased half time of clearance of an antigen (Soane, 1999: 55-65). It can be formulated in particles of different sizes where the antigen is incorporated (Illum, 1998: 1326 – 1331). Several studies showed the potential use of CS as an absorption enhancing agent for the administration of hydrophilic drugs. CS is able to reduce the transepithelial electrical resistance of a cultured intestinal epithelial cell line (Caco-2) (Schipper, 1997: 923–929; Opanasopit, 2007: 447–455; Artursson, 1994: 1358–1361). However, it has only limited ability for controlling the release of encapsulated compound due to its hydrophilic nature and easy soluble in acidic medium. Therefore, it needs to be crosslinked for drug delivery applications. To improve the drug delivery properties of CS, several modifications have also been studied. By simple covalent modifications of the polymer, its physicochemical properties can be changed and can be made for the peroral drug delivery purpose. Therefore, several CS derivatives have been synthesized in the last few years to obtain a modified carrier with altered physicochemical characteristics. Based on a literature search, it can be hypothesized that the transmucosal drug absorption enhancing properties of quaternized chitosans depend on molecular weight (MW), degree of quaternization (DQ), and other structural features (Colo, 2004: 2851–2862; Sandri, 2004: 351–359).

In this present study, natural polymeric microparticulate systems have been evaluated for their use as the carriers for oral vaccine delivery. In a preliminary study, Calcium-alginate (Ca-alginate) microparticles have been formulated for entrapment of the model protein bovine serum albumin (BSA) by electrospraying technique. Their physical, morphological properties, entrapment efficiency and release behavior have been investigated in accordance with formulation parameters. The optimized condition to produce the suitable size, shape, highest loading efficiency and sustained release was subsequently used for the entrapment of ovalbumin (OVA), a model antigen. Moreover, the desirable formulation of Ca-alginate was used to mix with yam starch and coated with chitosan derivatives to study their physical, morphological properties, entrapment efficiency, release behavior, mucoadhesive properties and cytotoxicity. Finally, the natural polymeric microparticulate systems were investigated for their *in vivo* immunological activity via oral vaccination.

1.2 Objective of this research

1. To fabricate natural polymeric microparticles by electrospraying method without using organic or acid solvents.
2. To investigate the influence of formulation factors such as type of natural polymers and amount of natural polymers on physicochemical properties, loading efficiency, and release characteristics of the microparticles.
3. To investigate *in vitro* bioadhesive properties and their cytotoxicity.
4. To investigate *in vivo* immunological activity of antigen loaded microparticles via oral vaccination.

1.3 The research hypothesis

1. Natural polymeric microparticles can be prepared by electrospraying method without using organic or acid solvents.
2. The type and amount of natural polymers blending influence the physicochemical properties, loading efficiency, and release characteristics of the microparticles.

3. The microparticles presenting in high loading efficiency, sustained release characteristics, good bioadhesive property and low cytotoxicity can improve oral vaccine delivery system.

CHAPTER 2
LITERATURE REVIEWS

2.1 Immune system

2.2 Vaccines

2.3 Adjuvant

- 2.3.1 Mineral salts
- 2.3.2 Oil emulsions
- 2.3.3 Bacterial derivatives
- 2.3.4 Virus like particles
- 2.3.5 Carbohydrate adjuvants
- 2.3.6 Immune stimulating complexes (ISCOM)
- 2.3.7 Liposomes
- 2.3.8 Polymeric microparticles

2.4 Mucosal vaccine delivery

- 2.4.1 Advantages of mucosal vaccination
- 2.4.2 M cell biology
  - 2.4.2.1 M cell structure and function
  - 2.4.2.2 General principles of M cell delivery
- 2.4.3 Oral vaccine delivery

2.5 Oral vaccines using polymeric microparticles

- 2.5.1 Biodegradable polymers
  - 2.5.1.1 Alginate
  - 2.5.1.2 Chitosan
  - 2.5.1.3 Starch
    - 2.5.1.3.1 Yam starch
- 2.5.2 Preparation of microparticles
  - 2.5.2.1 Solvent extraction and solvent evaporation
  - 2.5.2.2 Phase Separation
  - 2.5.2.3 Spray drying

2.1 Immune system

Immunity is defined as resistance to disease, specifically infectious disease. The collection of cell, tissues, and molecules that mediate resistance to infections is called the immune system, and the coordinated reaction of these cells and molecules to infectious microbes is the immune response (Abbas and Lichtman, 2009: 1-21). The immune system consists of a wide array of cells, soluble molecules, and tissues distributed all over the body. Surveillance and destruction of substances that are foreign substances are microorganisms such as bacteria, viruses, parasites, or fungi. However other substances that are foreign to the body are also eliminated by the immune system. The immune system is generally divided into two main categories, innate immunity and adaptive immunity (Figure 1).

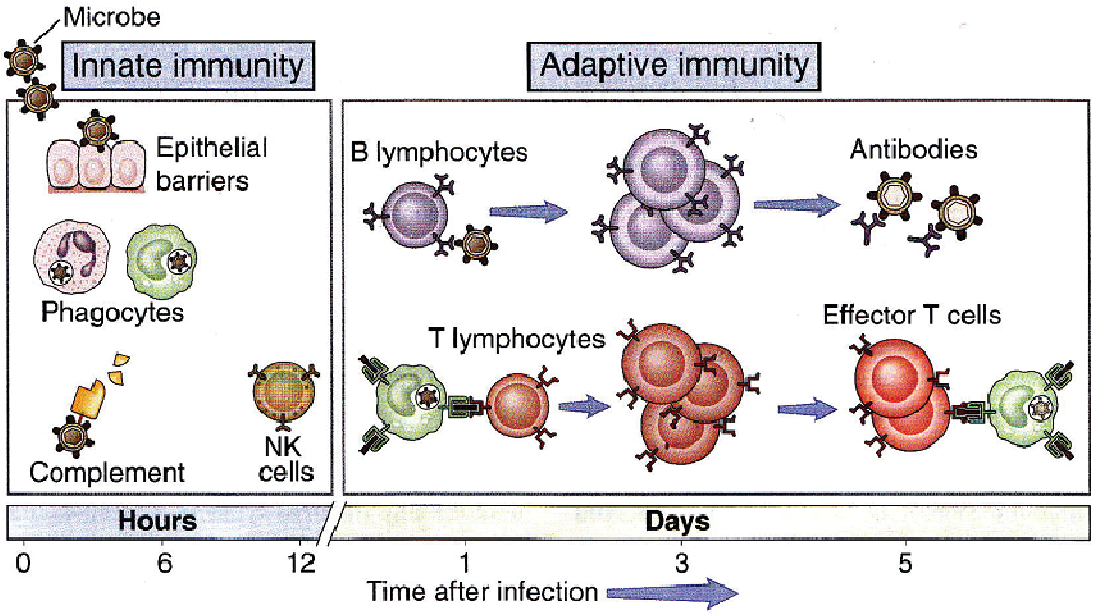

Figure 1 The principal mechanisms of innate and adaptive immunity.

Source : Abbas A. K. and Lichtman A. H. (2009). **Basic Immunology: Functions and Disorders of the Immune System.** 3rd ed. Philadelphia: Elsevier.

The term innate immunity also known as non-specific immune system first line of defense refers to the fact that this type of host defense is always present in healthy prepared to block the entry of microbes and to rapidly eliminate microbes that

do succeed in entering host tissue. Adaptive immunity is the type of host defense that is stimulated by microbes that invade tissues. It adapts to the presence of microbial invaders. The first line of defense in innate immunity is provided by epithelial barriers and by specialized cells and natural antibiotics present in epithelia, all of which function to block the entry of microbes. If microbes do breach epithelia and enter the tissues or circulation, they are attacked by phagocytes, specialized lymphocytes call natural killer cells, and several plasma proteins, including the proteins of the complement system. All of these agents of innate immunity specifically recognize and react against microbes but do not react against noninfectious foreign substances. Although innate immunity can effectively combat infections, many microbes that are pathogenic for human have evolved to resist innate immunity. Defense against these infectious agents is the task of the adaptive immune response, and this is why defects in the adaptive immune system result in increased susceptibility to infections. The adaptive immune system consists of lymphocytes and their products, such as antibodies. Whereas the mechanisms of innate immunity recognize structures shared by classes of microbes, the cells of adaptive immunity, namely, lymphocytes express receptors that specifically recognize different substances produced by microbes as well as noninfectious molecules. These substances are called “antigens” (Abbas and Lichtman, 2009: 1-21). The two types of adaptive immunity, humoral immunity and cell-mediated immunity are mediated by different cells and molecules and are designed to provide defense against extracellular microbes and intracellular microbes, respectively (Figure 2).

Humoral immunity is mediated by proteins called antibodies, which are produced by cells called B lymphocytes. Antibodies are secreted into the circulation and mucosal fluids. They neutralize and eliminate microbes and microbial toxins that are present outside of host cells in the blood and in the lumens of mucosal organs such as the gastrointestinal and respiratory tracts. One of the most important functions of antibodies is to stop microbes that are present at mucosal surfaces and in the blood from gaining access to and colonizing host cells and connective tissues. In this way, antibodies prevent infections from ever getting established. Antibodies cannot gain access to microbes that live and divide inside infected cells. Defense against such intracellular microbes is called cell mediated immunity because it is mediated by cells

call T lymphocytes. Some T lymphocytes activate by the phagocytes in to intracellular vesicles. Other T lymphocytes kill any type of host cells that are harboring infectious microbes in the cytoplasm. Thus, the antibodies produced by B lymphocytes recognize extracellular microbial antigens, whereas T lymphocytes recognize antigens produced by intracellular microbes. Another important difference between B and T lymphocytes is that most T cells recognize only protein antigens, whereas antibodies are able to recognize many different types of molecules, including proteins, carbohydrates, and lipids.

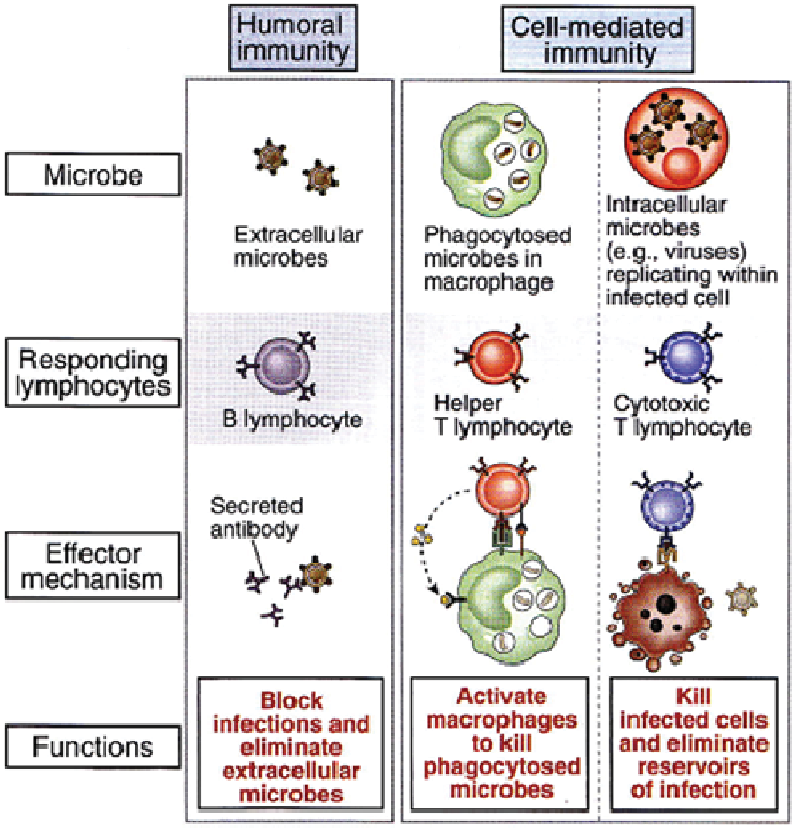

Figure 2 Type of adaptive immunity.

Source : Abbas A. K. and Lichtman A. H. (2009). **Basic Immunology: Functions and Disorders of the Immune System.** 3rd ed. Philadelphia: Elsevier.

An immune response to foreign antigen requires the presence of an antigen-presenting cell (APC), (usually either a macrophage or dendritic cell) in

combination with a B cell or T cell. When an APC presents an antigen on its cell surface to a B cell, the B cell is signaled to proliferate and produce antibodies that specifically bind to that antigen. If the antibodies bind to antigens on bacteria or parasites it acts as a signal for macrophages to engulf (phagocytose) and kill them. Another important function of antibodies is to initiate the "complement destruction cascade." When antibodies bind to cells or bacteria, serum proteins called complement bind to the immobilized antibodies and destroy the bacteria by creating holes in them. Antibodies can also signal natural killer cells and macrophages to kill viral or bacterial-infected cells. If the APC presents the antigen to T cells, the T cells become activated. Activated T cells proliferate and become secretory in the case of CD4+ T cells, or, if they are CD8+ T cells, they become activated to kill target cells that specifically express the antigen presented by the APC. The production of antibodies and the activity of CD8+ killer T cells are highly regulated by the CD4+ helper T cell subset. The CD4+ T cells provide growth factors or signals to these cells that signal them to proliferate and function more efficiently.

2.2 Vaccines

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease causing microorganism, and is often made from weakened or killed forms of the microbe. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters. History of vaccines was started when Edward Jenner began injecting an extract of cowpox lesions into patients to prevent smallpox infection in the late 18^{th} century, little could he have know how his crude inoculation would revolutionize the science of disease prevention and control. Since those humble beginnings, the science of vaccination has both spurred and adapted biotechnological advances to produce vaccines that are efficacious and safe (Kline, 1996: 349-379). There are two principles of vaccination 1. Passive vaccination: antibodies against the pathogen are administered. 2. Active vaccination: the immune system is confronted with alleviate pathogens and build up its own immune defense against the causative organism.

Vaccines have been divided in to three type namely 1) Live attenuated vaccines 2) Inactivated vaccines and 3) Subunit vaccines. Live attenuated vaccines are made up of living virus or bacteria that have been modified through a process to weaken (attenuate) and reduce its virulence. These wild viruses or bacteria are attenuated in a laboratory, usually by repeated culturing. A live attenuated vaccine creates a good immune response (cellular and antibody) and often provides lifelong immunity with only one or two doses. Inactivated vaccines do not contain live virus or bacteria. The diseases microbes have been killed by chemicals, heat or radiation. They cannot replicate and cause disease. Inactivated vaccines stimulate a weaker immune response than live vaccines and always require several doses to provide a protective immune response. Immunity can also diminish over time and a booster shot is required to maintain immunity. Inactivated vaccines can be composed of either whole viruses or bacteria, or fractions of either. Subunit vaccines are antigenic proteins and polysaccharides or recombinant protein components that best stimulates the immune system. Because these vaccines only use a part and not all the other molecules that make up the microbe, the chances of adverse reactions to the vaccine are lower, for similar reasons, may require adjuvants or multiple dosing strategies to induce protection.

2.3 Adjuvant

The ultimate principle of vaccination is to generate a strong immune response against infectious diseases (Aguilar and Rodriguez, 2007: 3752–3762). An adjuvant is a compound administered with the antigen, or one that provides a mode of presentation of antigen that will enhance the immune response to ward that antigen. The mechanisms of adjuvant action are complex, and as more is discovered about their effects on the immune system, the less likely it appears that a few theories will be able to explain the action of the entire spectrum of adjuvant-antigen combinations. For simplicity, adjuvant action may be broken down into two general categories: (a) direct stimulatory effect on the immune system and (b) the method of antigen presentation to the immune system (Sivakumara, 2011: 197–206). However, many adjuvants do not appear to have direct interactions with immunocompetent cells. Rather, these adjuvants are hypothesized to act by affecting the manner in which the

immune system interacts with antigen. Generally, adjuvants can be used for various purposes (Aguilar and Rodriguez, 2007: 3752–3762): 1. To augment the immune response of any antigens by delivering in native form. 2. To reduce the multiple immunization protocol for protective immunity. In particular to develop single step vaccination coverage that can reduce the vaccination costs. 3. To enhance the immune response of immune compromised adults and weakened immune system of children, to elicit cytotoxic T lymphocytes response and generate local immune response (Achal, Nicholas and Jerry, 2005: 63-68). There are several types of adjuvants with differing modes of action. These include mineral salts, oil emulsions, immune bacterial derivatives, stimulating complexes (ISCOM), carbohydrate adjuvants, liposomes, virus like particles and polymeric microparticle adjuvants.

2.3.1 Mineral salts

Mineral salts such as alum and calcium phosphate have been used as adjuvant in vaccine formulations (Table 1). Aluminum-containing compounds, such as aluminum hydroxide, are the adjuvants most widely used in human vaccines and the only ones currently approved by the FDA. Antigen is adsorbed onto an aluminum hydroxide suspension and is thus injected in the context of an antigen-aluminum complex. Aluminum adsorbates have been hypothesized to work primarily by a “depot” effect by which adsorbed antigen is kept at the injection site (Glenny, Buttle and Stevens, 1931: 267-275) or within the peripheral lymph nodes for extended periods (Bomford, 1988: 35-41) and becomes available for more efficient processing by immunocompetent cells. Even though alum has good properties it is not much suitable for small proteins because the alum adsorbed vaccines elicit a short term immune response requiring many boosters for attaining minimum optimal threshold immune response. Consequently, it is necessary to find a new adjuvant that can replace the alum type adjuvant.

2.3.2 Oil emulsions

Le Moignic and Piony (Moignic and Piony, 1916: 352-358) demonstrated that the suspension of killed *Salmonella typhimurium* with mineral oil emulsion elicited increased immune response. Later, Freunds et al. (1937) prepared a water-in-oil type of emulsion adjuvant using paraffin oil mixed with killed Mycobacteria called

Freund's complete adjuvant (FCA) and without Mycobacteria referred as Freund's incomplete adjuvant (FIA). FIA consists of aqueous antigen emulsified in a low-viscosity, low specific gravity mineral oil with and added emulsifier (Edelman, 1980: 370-383). FIA is hypothesized to act in a manner similar to aluminum compounds, but its superior adjuvanticity may be due to several factors. The depot effect of FIA is apparently more prolonged than that of alum. Because antigen may be released from within water-in-oil emulsion slowly more than from alum (Herbert, 1968: 301-318). Additionally, the nonmetabolizable mineral oil component of FIA is more inflammatory than alum and, thus, may be more potent in its stimulation of immune cells. The importance of this aspect of FIA is demonstrated when other metabolizable oils, such as peanut oil, are substituted for mineral oil; both the adjuvanticity and the intensity of local reactions were reduced (Fukumi, 1967: 237; Harris, 1969: 617-621; Stevens, 1993: 176-188). However, FIA is not currently used due to poor immunogenic effect, induces local irritation, may cause granuloma and cystformation at the site of injection and reported carcinogenic in mice (Murray, Cohen and Hardegree, 1972: 146–151; Gupta et al., 1993: 293–306). MF 59 is an adjuvant developed by the ex-Chiron now Novartis Vaccines (O'Hagan, Wack and Podda, 2007: 740–744). It consists of oil-in-water emulsion, comprising 4.3% of squalene oil as dispersed phase, which is stabilized by two non-ionic surfactants (Tween 80 and Span 85), and a low ionic strength citrate buffers as continuous phase. MF 59 is originally developed as delivery agent that contains muramyl tripeptide phosphatidylethanolamine (MTP-PE), which activates non-Toll-Like receptors (TLR) sensing receptors known as NOD LRRs (Akira, Uematsu and Takeuchi, 2006: 783–801) that leads toxicity. But the MF 59 without MTP-PE proved as a potent adjuvant for influenza vaccine (Fluad) tested in animal model and later human trials have been shown successful results. Thus, it becomes the first new adjuvant for human use after alum (Tagliabue, 2008: 347–349). The properties of oil emulsion as vaccine adjuvant were summarized in Table1.

2.3.3 Bacterial derivatives

Bacterial toxins and toxoids from *Escherichia coli* and *Vibrio cholera* have been reported as mucosal adjuvants (Rappuoli et al., 1999: 493-500). Bacterial cell

wall components like peptidoglycan or lipopolysaccahrides (LPS) enhance the immune response by mediating through activation of Toll-like receptors (TLR) that activates the host immune system (Holmgren and Czerkinsky, 1992: 387-391). These toxins are composed by an A subunit that has enzymatic activity (ADP-ribosyl transferase) and a B subunit, formed by five identical units, that is responsible for binding to the cell membrane (Yamamoto, 1997: 1203-1210). Cholera toxin (CT) and LT (produced by *Escherichia coli*) are still among the most powerful mucosal adjuvants and they are used as gold standards in experiments measuring adjuvant efficacy. They induce strong and long lasting systemic and mucosal response and potentiate the response to the antigen at low concentration and with a low number of doses (Pizza et al., 2001: 2534-2541; Cooper, 1995: 559-580). The strong toxicity of these molecules does not allow their use in human, therefore several mutants of CT and LT with reduced or abolished toxicity have been developed in the latest years (Cooper, McComb and Steele, 1991: 408-415; Sheets, Unger and Giggleman, 1991: 41-45). An example of microbial derivatives as vaccine adjuvant was shown in Table 1.

2.3.4 Virus like particles

Virus-like particles (VLPs) are a highly effective type of subunit vaccine that mimic the overall structure of virus particles without the requirement of containing infectious genetic material. Indeed, many VLPs completely lack the DNA or RNA genome of the virus and thus have the authentic conformation of viral capsid proteins seen with inactivated and attenuated virus vaccines, without any of the drawbacks of reversion, recombination and re-assortment. In practical terms, that VLPs mimic the structure of virus particles usually means that lower doses of antigen relative to subunit vaccines are sufficient to elicit a similar protective response. In addition to their ability to stimulate B-cell-mediated immune responses, VLPs have been shown to be highly effective at stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses (Paliard et al., 2000: 273-282; Murat et al., 2003: 6753-6758; Antonis et al., 2006 5481-5490). This feature of VLP vaccines is likely to be a major contribution to their effectiveness. The VLP presents the antigens are capably engaged by dendritic cells that induce strong immune response (Young et

al., 2006: 5406-5421; Dell et al., 2006: 2238-2247; Aguilar and Rodriguez, 2007: 3751-3762). Recombinant hepatitis B surface antigen (HBsAg) has been produced as VLPs in *Saccharomyces cerevisiae*. Recently, human papilloma virus (HPV) vaccine has been produced by this VLP technology and approved by US Food and Drug Administration for clinical use (Srivastava and Singh, 2005: 15-28).

2.3.5 Carbohydrate adjuvants

Several complex carbohydrates of natural origin such as gamma inulin derived from plant root of the composite family stimulate both humoral and cellular immunity. It activates complement pathway and increases the production of activated C3 and thereby activating macrophages (Gregoriadis, 1990: 89-97). Gamma inulin can be combined with variety of other adjuvants to get a better adjuvant, for example algammulin. Algammulin is a combination of Gamma inulin with aluminum hydroxide a potent inducer of Th1 and Th2 activity when compared to gamma inulin alone (Harokopakis, Hajishengallis and Michalek, 1998: 4299-4304). Other polysaccharides such as glucans, dextrans, glucomannons, galactomannans, levans and xylans also enhance the immune response (Childers, 2000: 5509-5516).

2.3.6 Immune stimulating complexes (ISCOM)

Immunostimulatory complexes (ISCOMs) are particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin (Lovgren, 1988: 161-172). A description of ISCOMs can be found in a report dating back to 1973, but at that time their potential as subunit vaccine delivery systems was not recognized. The initial use of ISCOMs came from Morein by demonstrating that formulating micelles of the saponin Quil A extracted from the bark of Quillaja saponaria with viruses resulted in an effective particulate vaccine (Morein et al., 1984: 457-460). This compound has been further modified to produce a triterpenoid component, QS21, and the cage-like structures known as immunostimulating complexes (ISCOM) consists of an antigen, cholesterol, phospholipid and a saponin derivative (Guy, 2007: 505-517; Cox et al., 2006: 511-539). ISCOM combine the advantages of a particulate carrier system with the presence of an inbuilt adjuvant (Quil A) and consequently have been found to be more immunogenic than other colloidal systems such as liposomes and protein micelles (Sanders, 2005: 119-128). These adjuvants activate

CD8+ cytotoxic T cells and generally will stimulate a Th1 type response, but can also induce the production of both Th1 and Th2 cytokines depending on the incorporation of different antigens or adjuvants (Helgeby et al., 2006: 3697-3706; Walker, 1994: 387-400). The properties of ISCOM as vaccine adjuvant were shown in Table 1.

2.3.7 Liposomes

Liposomes are bilayer membrane vesicles composed of phospholipids and cholesterol that were originally formulated as carriers for drugs (Allison and Gregoriadis, 1974: 252-258). Liposomes are uni- or multilamellar. Charge can be added by incorporation of charged lipids and rigidity by using lipids with different phase transition temperatures. Concentric layers of phospholipids with aqueous phases in between form multilamellar vesicles (MLV) with an average size of 1–50 mm. When phospholipids are mixed with water under low shear conditions, MLV usually spontaneously form. Liposomes can also be prepared with a single bilayer as either large unilamellar vesicles (LUV) with a 100–500 nm size range or small unilamellar vesicles (SUV) with a 25–100 nm size range. Preparation of empty liposomes is easy, but efficient incorporation of antigen may require extensive optimization programmes. Liposomes are useful as carriers of antigens for eliciting protective immune responses against bacterial, viral, and parasitic infections. The antigen can be either integrated or bound to their surface. Their efficacy for mucosal vaccination is increased when an adjuvant is coformulated or expressed on their surface (Fries et al., 1992: 358-362; Baca-Estrada, Foldvari and Snider, 1999: 455-462). Liposomes can induce both humoral and cellular immunity to protein and polysaccharide antigens (Owis, 2000: 3946-3956). For example, a liposomal malaria vaccine containing a particular epitope from *Plasmodium falciparum* was evaluated in a clinical study (Zheng et al., 1999: 1011-1020) and was shown to be safe and effective. Baca-Estrada et al. also demonstrated the feasibility of using multilamellar vesicles and biphasic delivery systems as carriers for the bovine herpes virus type-1 glycoprotein D (antigen), and IL-12 or IL-4 (cytokine adjuvants) to achieve antigen-specific cellular and humoral immune responses in mice (Schirmbeck, 1996: 111-119). The liposomal based vaccines fuse with cell membranes of macrophages, enabling the delivery of proteins into the cytoplasm and enter in to MHC class I

pathway and activate CD8 cells (Paliard et al., 2000: 273-282; Murata et al., 2003: 6753-6758). However, the major drawbacks of liposomes are manufacturing difficulties such as stability, high cost and may produce pain at the site of injection (Table1).

2.3.8 Polymeric microparticles

Polymeric microparticles are solid, spherical with approximately spherical particles ranging in size from 1 to 1000 µm. Active substances may be either adsorbed at the surface of the polymer or encapsulated within the particle. Polymeric microspheres have shown great potential as a next generation adjuvant to replace or complement existing aluminum salts for vaccine potentiation (Aguilar and Rodriguez, 2007: 3752-3762). Biodegradable polymers are being used as sutures and drug carriers, because of the biocompatible, nontoxic nature and their biodegradable properties. The polymers which are chosen as excipients (adjuvants) for parenterally administered vaccines should meet some of the requirements, including being biodegradable, safe, antigen compatible and permeable, stable *in vitro* easy to process, alone responsible formulations and ideally inexpensive. The biodegradable synthetic and natural polymers have been investigated for the control release of macromolecular drugs and proteins. These polymers in the form of microspheres/microparticles seem to be preferred for better controlled release of antigen (Table 1).

Table 1 Properties of mineral salts, oil emulsion, microbial derivatives and particulate delivery vehicle as vaccine adjuvant.

Adjuvant type	Representing examples	Mechanism of action	Advantage	Disadvantage
Mineral salts	Aluminum salts-aluminum hydroxide or aluminum phosphate	1. Sort term depot effect 2. Induction of cytokine network 3. Complement activation 4. Delivery of antigens to different APC 5. Strong Th2 response	1. Inexpensive 2. Safe 3. Efficient uptake of alum adsorbed antigens by antigen presenting cells due to particulate nature with optimum size 4. Long lasting immune response when compared to soluble antigens	1. Not suitable for recombinant proteins and subunit vaccines 2. Adverse effects have been reported such as inflammation and stimulate local production of erythema, granuloma, contact hypersensitivity and IgE mediated hypersensitivity. 3. Alum cannot be frozen or lypholized 4. Unable to induce cytotoxic T cell response
	Calcium salts	1. Short term depot effect 2. Adsorbs soluble antigens and presents them in a particulate form to the immune system	It is a normal constituent of the body and therefore well tolerated, elicit high IgG type antibodies	1. It lacks immunomodulatory response 2. Neurological reactions have been observed 3. Local irritation and active inflammatory reaction at the site of injection

Table 1 Properties of mineral salts, oil emulsion, microbial derivatives and particulate delivery vehicle as vaccine adjuvant (continue).

Adjuvant type	Representing examples	Mechanism of action	Advantage	Disadvantage
Oil emulsion	Freund's complete adjuvant (FCA)	1. Short term depot effect 2. Strong Th1 and Th2 response by producing	Strong immune response	Highly toxic
	Freund's incomplete adjuvant (FIA)	1. Short term depot 2. It induces weak Th1 and Th2 response	Lesser side e□ects than FCA	1. Poor Immuno-modulatory effect 2. Local irritant effect may induce granuloma and cyst formation
	MF 59	Inducing local immune stimulatory e□ect at the site of injection, regulates cytokines, chemokines, recruitment of CD11b+, MHC II+ cells and enhance antigen uptake by dendritic cells	It is a superior than alum adjuvant for influenza vaccine	1. Pain at injection site 2. Reactogenicity 3. Induces inflammatory arthritis
Microbial derivatives	ASO4	Local activation of NF-kB activity, cytokine production, optimal activation of APC and generation of Th1 response	It is superior than alum adjuvant for cancer, HBV, malaria and HPV	1. Reactogenicity 2. Issues on consistency of preparation of vaccines from bacteria 3. Cost effective

Table 1 Properties of mineral salts, oil emulsion, microbial derivatives and particulate delivery vehicle as vaccine adjuvant (continue).

Adjuvant type	Representing examples	Mechanism of action	Advantage	Disadvantage
Particulate delivery vehicle	ISCOM	1. Generate CTL response, induce cytokines 2. Directly phagocytosed by macrophages	Induces strong immune response	1. Severe pain at the site of injection 2. Severe toxicity includes severe haemolysis, granulomas
	Liposomes	Fuse with cell membrane of macrophages, enable antigen in to the cytoplasma, enter MHC class I path way and activate CD8 CTL response	Induces strong immune response	1. Manufacturing difficulties due to stability 2. High cost 3. Severe pain at the site of injections
	Polymeric microparticle	1. Long term depot effect from weeks to months 2. Pulsatile release of antigens 3. Target to antigen presenting cells	1. It can mimic the priming and boosting effect of conventional vaccine 2. Potential for single shot vaccines and can reduces the cost of vaccination	1. Issues on stability of antigens during micro encapsulation and storage 2. Issues on dose optimization

Source : Sivakumar, S.M. et al. (2011). "Vaccine adjuvants – Current status and prospects on controlled release adjuvancity." **Saudi Pharmaceutical Journal** 19, 4: 197–206.

A vaccine can work the most efficiently, if it is able to elicit both humoral and cellular immune responses. However, conventional vaccines often induce antibody responses with only limited cellular immunity (Russell-Jones, 2000:49-54). Many of the vaccines currently in use are directed towards systemic pathogens or toxins and are administered by systemic injection (e.g., intramuscular or subcutaneous) (Wang et al., 2004: 6357-6365). This will most likely elicit a strong systemic immune response (e.g., production of antibodies) but only weak mucosal immunity (Holmgren and Czerkinsky, 2005: 45-53). In spite of these considerations, most of the vaccines so far in use are parenteral vaccines and the few available mucosal vaccines are mainly based on whole microorganisms that act as potent immunogens (Magistris, 2006: 52-67). However, to improve safety modern vaccines are mainly based on pathogen derived purified antigens. These are generally poorly immunogenic and need specific mucosal adjuvants that elicit or amplify the immune response to them (McGhee et al., 1992: 75-88).

2.4 Mucosal vaccine delivery

The majority of pathogens initiate infection by interacting with host mucosae. After colonization, some microorganisms generate disease by releasing toxic factors, others penetrate into host tissues and give systemic or organ-specific diseases. Thus, stimulation of mucosal defenses is very important for controlling infection and preventing disease. Induction of local responses is possible through the use of mucosal vaccines as parenteral vaccination elicits little or no IgA, the antibody isotype that is abundantly secreted in mucosal tissues and that is responsible for their defense (Neutra and Kozlowski, 2006: 148-158). The injection of vaccine antigens may lead to the production of measurable quantities of antigen-specific antibodies and an increase in numbers of lymphoid cells in the blood. This response can be effective against some pathogens that enter the host via the mucosa, but less effective against other mucosal pathogens, such as HIV, that required layers of protection on both sides of the epithelial barrier (Chadwick, Kriegel and Amiji, 2010: 394-407). In contrast, mucosal vaccination via the oral or intranasal route generally favors the development of mucosal antibody and cell-mediated immune response (Neutra and Kozlowski, 2006: 148-158). This mandates successful vaccine delivery to the mucosal site of

interest in order to produce a strong protective effect, which introduces several distinct challenges in the development of an efficacious vaccine (Levine, 2003: 99-103).

2.4.1 Advantages of mucosal vaccination

The characteristics of the immune responses induced through the mucosal route confer to mucosal vaccination several advantages over parenteral vaccination. First, mucosal immunization elicits antigen-specific IgA antibodies at the site of infection. This local pathogen-specific response may be important not only for the prevention of infectious diseases in the vaccine recipient, but may be also useful to eliminate the healthy carrier condition and the consequent transmission of infections to non-protected individuals. Second, because of the expression of mucosa-specific homing receptors by mucosal primed lymphocytes, the immunization at one mucosal site can induce specific responses at distant sites. Third, in addition to IgA responses, mucosal vaccination induces systemic IgG responses that represent a further defense against invasion by microorganisms or their products. The simultaneous stimulation of mucosal and systemic antibodies is particularly important for protection against pathogens such as HIV that can infect host through both systemic and mucosal route. Fourth, in addition to serum IgG and mucosal IgA antibodies, mucosal immunization can stimulate cell-mediated responses including helper CD4+ T cells and CD8+ cytotoxic T lymphocytes, the latter being important to combat intracellular pathogens. Thus, mucosal vaccines have the potential to activate all the different arms of the immune system. Mucosal administration of vaccines also offers a number of important practical advantages. First of all it is non-invasive and does not require the use of needles. This would increase vaccine compliance and would also avoid problems of blood transmissible infections in developing countries due to needle re-use (Gebert, 1996: 91-159). Moreover, mucosal vaccination is relatively easy and does not require expensive specialized personnel. Reduced adverse effects and the potential for frequent boosting may also represent further advantages over injectable vaccines. Finally, production of mucosal vaccines may be less expensive than that of injectable vaccines that require high standards of purity, in addition to sterility.

2.4.2 M cell biology

Potential sites for mucosal drug and vaccine delivery include the alimentary, respiratory and urogenital tracts and the eye. The epithelia at these sites generally constitute effective barriers which prevent the uptake of microorganisms and other particulates. Absorption at these sites is further restricted by fluid secretion which dilutes and may flush away applied delivery vehicles. However, mucosal sites also include the organized mucosa-associated lymphoid tissues (O-MALT) which are the specialized antigen sampling sites of the mucosal immune system. The antigen sampling function of the O-MALT is performed predominantly by the membranous epithelial M cells. While these cells are specialized for antigen sampling they are also exploited as a route of host invasion by many pathogens[62-66]. In addition, M cells represent a potential portal for mucosal drug and vaccine delivery since they possess a high transcytotic capacity and are able to transport a broad range of materials including particulates (Ermak and Giannasca, 1998: 261–283; Clark, Hirst and Jepson, 2000: 207-223; Gebert, Rothkotter and Pabst, 1996: 91-159).

2.4.2.1 M cell structure and function

The intestinal epithelial barrier is composed of a single layer of epithelial cells which predominantly consists of enterocytes interspersed by mucus secreting goblet cells. The epithelial cells are sealed at their apical membranes by tight junctions and, while cells are constantly extruded into the gut lumen, epithelial integrity is maintained by cell replacement from the crypts. O-MALTs are located throughout the gastrointestinal tract and consist of lymphoid follicles arranged either singly or as clusters to form distinct structures such as the Peyer's patches and appendix. The epithelium overlying the lymphoid follicles is termed the follicle-associated epithelium (FAE) and is distinguished from the intestinal epithelium at other sites chiefly by the presence of the specialized antigen sampling M cells (Figure 3A). Together, the FAE, lymphoid follicles and associated structures form the antigen sampling and inductive sites of the mucosal immune system. Antigen sampling (and potential drug and vaccine delivery) is thought to be facilitated by both these features. The sparsity of the microvilli renders the M cell apical membranes relatively accessible to reagents within the gut lumen. After M cell adhesion, these agents need

only be transported a short distance across the thin M cell cytoplasmic rim before reaching the M cell pocket and underlying lymphoid cells, a feature which permits rapid delivery of vaccine antigens directly to the inductive O-MALT sites.

2.4.2.2 General principles of M cell delivery

Antigen sampling (and potential drug and vaccine delivery) by M cells is facilitated by the relative accessibility of the M cell apical membranes (Figure 3B). Both secretory IgA and mucus are present in reduced quantities at the surface of the FAE compared to other intestinal epithelial sites, the M cell microvilli are relatively sparse and irregular, and the M cell surface glycocalyx is relatively thin. To achieve effective delivery, the administered reagent should ideally target to and exhibit high levels of binding to the M cell apical membranes, and subsequently be internalised and transported in an active form to the M cell pocket. M cells possess a high transcytotic capacity, and are able to transport a variety of materials including macromolecules, inert particles and microorganisms (Yang, Peters and Williams, 2008: 239-247). Moreover, they appear to be the most efficient cell type for the intestinal absorption of biologically active reagents administered in particulate form, a feature which presumably reflects their specialised antigen sampling function.

Another mechanism by which antigens may be captured at the mucosal surface has been proposed following the *in vitro* observation that dendritic cells form tight junctions with polarized epithelial cells. This allows these professional antigen presenting cells to extend dendrite-like processes through the epithelial cell monolayer and to sample luminal antigens directly. In addition to dendritic cells, in the lamina propria of the epithelia there are other immunocompetent cells such as macrophages, B and T lymphocytes and plasma cells. Antibodies produced in the lamina propria of epithelia are transported to the mucosal surface by epithelial cells through a basolateral to apical receptor-mediated transcytosis. The antibody isotype that is most abundantly secreted at mucosal surfaces is IgA. Mucosal IgA are dimeric immunoglobulins called “secretory IgA” and have several important functions in host defense. First of all, they neutralize microbes and their products, thus avoiding mucosa colonization and invasion by pathogens. Furthermore, they have been shown to neutralize bacterial products or viruses within the epithelial cells, during their

transcellular transport. Finally, IgA that are present in the lamina propria can capture antigens such as toxins and viruses and are able to excrete them from the mucosal tissue by intracellular cycling (McGhee et al., 1992: 75-88).

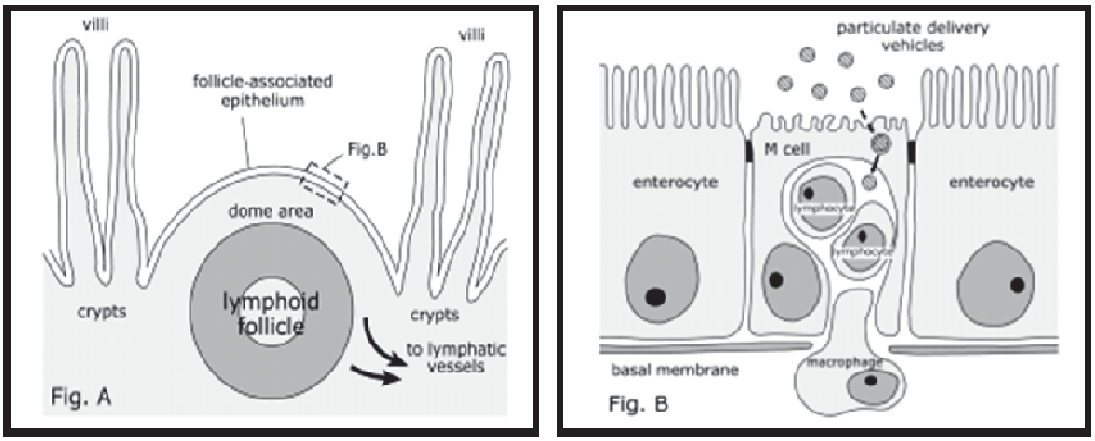

Figure 3 Schematic transverse sections of a Peyer's patch lymphoid follicle and overlying follicle-associated epithelium (FAE), depicting M cell transport of particulate delivery vehicles. The general structure of intestinal organised mucosa-associated lymphoid tissues (O-MALT) is represented by the schematic transverse section of a Peyer's patch lymphoid follicle and associated structures in (A). The lymphoid follicle is situated beneath a dome area which protrudes into the gut lumen between villi and which is covered by the follicle-associated epithelium (FAE). This epithelium is characterised by the presence of specialised antigen sampling M cells (depicted in B)

Source : Des Rieux, A. et al. (2006). "Nanoparticles as potential oral delivery systems of protein and vaccines: A mechanistic approach." **J Control Release** 116, 1-27.

2.4.3 Oral vaccine delivery

The oral route of administration is most commonly used for delivery of vaccines (Russell-Jones, 2000: 49-54). Because this route of vaccine delivery has added potential advantages in that it may be used for immunization against systemic infections, it is easy to administer, has low cost, increased patient comfort and

increased patient compliance. Vaccines that have been developed for oral administration include viral vaccines against polio and rotavirus, bacterial vaccines against typhoid fever and cholera, live attenuated bacteria expressing foreign antigens, and edible transgenic plant vaccines, among others. However, oral vaccine delivery is presented with the major challenge of antigen uptake since the intestine is designed to prevent invasion of foreign molecules via tight epithelial junctions and high levels of enzymatic activity (Webster, 2003: 277-240). As a result, many orally administered vaccines are degraded in the gastrointestinal tract and require a higher dose of antigen or several repeated doses to supply sufficient antigen for induction of immune protection (O'Hagan, 1998: 305-320). Large doses of orally administered antigen can lead to a short lived secretion of antigen specific IgA and the development of systemic tolerance. In addition, an antigen specific serum antibody response will not be induced (Webster, 2003: 277-240). Thus, the type of vaccine, route of vaccine delivery and susceptibility to environmental factors will have significant implications to the overall success of a vaccine. In order to improve the efficiency of mucosal vaccines, particle mediated delivery systems can be employed. An ideal particulate delivery system for mucosal vaccination should be able to target the necessary mucosal region, allow interaction with the cells of interest, and provide protection of the vaccine antigen during delivery.

2.5 Oral vaccines using polymeric microparticles

Polymeric particles used for vaccines delivery are defined as colloidal systems made of solid polymers that may be classified according to their size and preparation processes. The potentials and pitfalls of microsparticles are shown in Table 2.

The term of microparticles designates systems larger than 1 μm. Microparticles are composed of a polymeric wall containing a liquid inner core where the drug is entrapped while microspheres are made of a solid polymeric matrix in which the drug or antigen can be dispersed. Active substances may be either adsorbed at the surface of the polymer or encapsulated within the particle. Regardless of the preparation process, the ultimate physicochemical properties of the particles are greatly influenced by the experimental conditions of manufacturing. For example,

stirring mode and speed greatly influence the particle size and, similarly, the solvent elimination process will affect the hardening process and the final morphology of the particles. Microparticles may be produced by polymerization of synthetic monomers, or dispersion of synthetic polymers or natural macromolecules.

Table 2 The potentials and pitfalls of microparticles delivery systems

Potentials	**Pitfalls**
Protection of antigen during transit	Poor uptake of microparticles
Controlled release of antigen	Potential loss of antigenicity during encapsulation and storage
Potential for pulsed release	Variability of uptake among individuals
Incoporation of multiple antigens and immunostimulants possible	Expensive and complicated manufacturing process
Polymer can enhance immunogenicity of synthetic subunit vaccines	Toxicity of residual organic solvents

Source : Kline, D.F., Hanes, J. and Langer, R. (1996). "Oral vaccination by Microspheres" In **Microparticulate systems for the delivery of proteins and vaccines**, 381-397. Edited by Bernstein, H. New York: Marcel Dekke.

Microparticles protect the antigens from degradation and promote interaction with the mucosal epithelium. Their uptake by M cells favors antigen targeting to Peyer's patches in the intestine following oral administration (Eldridge et al., 1989: 191-202; Lutsiak et al., 2002: 1480-1487). In addition, it has been observed that microparticles of appropriate size can be taken up by antigen presenting cells such as dendritic cells *in vitro* and *in vivo* (Newman et al., 2002: 480-486; O'Hagan and Singh, 2003: 269-283). The antigen can also be adsorbed onto the surface of microparticles, a process that is used when microencapsulation can damage the antigen molecule (Okada and Toguchi, 1995: 1-99). Microparticles have been used in several models of experimental vaccination including DNA vaccines. Furthermore, Poly(Lactide-co-Glycolide) (PLG) microparticles have already been used in humans as controlled release drug delivery systems and for other biomedical purposes (Putney

and Burke, 1998: 153-157; Katz et al., 2003: 341-346) and clinical trials have been performed with oral vaccines incorporated in PLG microparticles (Okada and Toguchi, 1995: 1-99).

2.5.1 Biodegradable polymers

When attempting to design a microparticulate antigen delivery system for targeting mucosal sites, it is important to choose a polymer that is biodegradable, biocompatible, and safe for use in humans. Implantable polymer have frequently been used for various application where localized delivery of drug is desired, but the non-biodegradable systems need to be surgically removed from the body once complete release has occurred. A biodegradable polymer is ideal for immunization purposes, for it can release the antigen at the desired rate and does not necessitate and additional surgical step for retrieval of the depleted system. Biodegradable polymers should be 1) non-toxic, 2) capable of maintaining good mechanical integrity until degraded, and 3) capable of controlled rates of degradation. Biodegradable polymers can be either natural or synthetic.

In the design of oral delivery of peptide, protein or antigen, pH sensitive hydrogels have attracted increasing attention. Swelling of such hydrogels in the stomach should be minimal and thus the drug release will also be minimal. The extent of swelling increases as the hydrogels pass down the intestinal tract due to an increase in pH. A variety of synthetic or natural polymers with acidic or basic pendant groups have been employed to fabricate pH sensitive hydrogels. Alginate, chitosan and starch are naturally occurring biopolymers that are finding widespread applications in food and pharmaceutical industry. Alginate is used extensively in food industry as a thickener, emulsifier and as a stabilizer. Chitosan is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity. This review describes the sources, physical and chemical properties of these polymers that enable them to become suitable for protein delivery, the mechanisms of hydrogel formation, modifications which increase their protein encapsulation efficiency and the recent trends in their application. Starch is one of the most important natural organic compounds, abundant in nature. Over time, demand for starch by non-food industries will tend to grow faster than demand for starch by food industries. In recent years,

pharmaceutical companies widely use starches in various stages of drug development technology. Excipient plays a very important role in solid dosage formulation by imparting mechanical strength, stability and tablet disintegration.

2.5.1.1 Alginate

Commercial alginates are extracted from three species of brown algae. These include *Laminaria hyperborean*, *Ascophyllum nodosum*, and *Macrocystis pyrifera*; in which alginate comprises up to 40% of the dry weight (Sutherland, 1991: 309-331; Rees and Welsh, 1977: 214-224). Alginate is a water soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1–4 linked α-L-guluronic and β-D-mannuronic acid residues. Figure 4 shows the structures of mannuronic and guluronic acid residues and the binding between these residues in alginate. The gelation of alginate can be carried out under an extremely mild environment and uses non-toxic reactants. The most important property of alginates is their ability to form gels by reaction with divalent cations such as Ca^{2+}. Alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, in to a divalent crosslinking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. Monovalent cations and Mg^{2+} ions do not induce gelation (Dupuy, Arien and Minnot, 1994: 71-82). The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure shown in Figure 5. The divalent cations bind to the α-L-guluronic acid blocks in a highly cooperative manner and the size of the cooperative unit is more than 20 monomers (Sutherland, 1991: 309-331). Each alginate chain dimerizes to form junctions with many other chains and as a result gel networks are formed.

Protein entrapped alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, in to a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. The biological activity of drugs can be retained in the calcium-cross-linked alginate encapsulation process. Alginate obtained a ‘‘generally recognized as safe’’ status as a food and pharmaceutical ingredient by the U.S. Food and Drug Administration (FDA) in the early 1970s. It is generally regarded as a nontoxic and nonirritant material (Aguilar and Rodriguez, 2007: 3752-

3762). It poses many ideal characteristics which make it suitable polymer to use in vaccine developments, e.g. non-immunogenisity, stability with long shelf life, biodegradability and cheap to produce (Fundueanu et al., 1998: 11-21). Recently, alginate in form microspheres are used in several studies and showed to be potentially a suitable vaccine delivery system and immunoadjuvant (Lemoine et al., 1998: 9-19; Tafaghodi et al., 2006: 37-43; Mittal et al., 2000: 253-263). Immunization of animals by alginate microspheres containing antigenic proteins elicited both humoral and cell-mediated immune responses (Dobaknti et al., 2009: 296-310). The adjuvant effect of alginate was shown in Dobakhti et al. (Dobaknti et al., 2009: 296-310). This studied, the immune responses induced by subcutaneous administration of BCG plus alginate were significantly better than the responses induced by standard BCG vaccination (Otterlei et al., 1993: 19177-1925). Higher allergen-specific total IgG but much lower amounts of IgE were induced with the alginate conjugated extract than with unmodified extract. There are also some studies that showed the mannuronic acid rich alginates have immunostimulating properties itself, stimulating monocytes to cytokine production (Flo et al., 2002: 35489-35495) in where the TLR2 and TLR4 seemed to be involved (Banensee and Paranjpe, 2005: 503-510). In a more recent study (Roberts, 1992: 274-315), the effect of bio-materials on dendritic cell maturation was evaluated and it was demonstrated that the expression levels of CD86, CD40 and HLA-DQ (MHC class II) molecules, indicative of dendritic cell maturation, were decreased in the presence of alginate. The review results indicate that the alginate preparations can be used for mucosal as well as for parenteral treatment. This makes these particles interesting for application in diseases like viral infections, cancer or allergy.

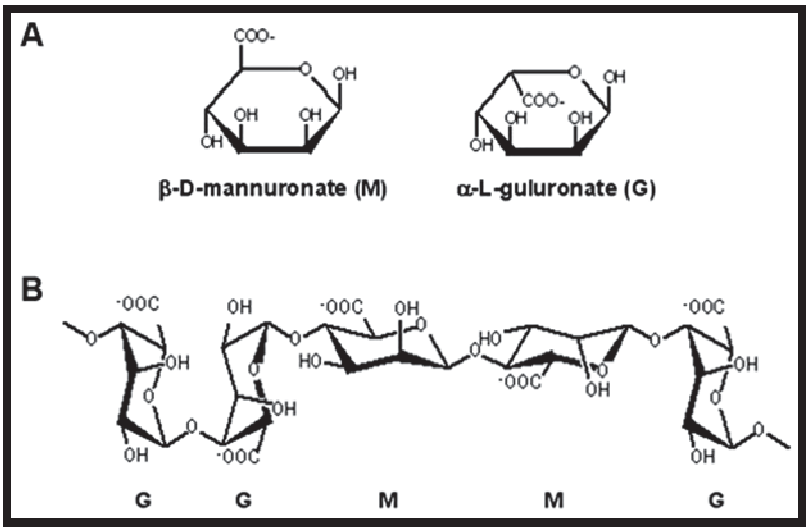

Figure 4 Chemical structure of alginate (a) the monomers in alginate; (b) the alginate chain

Source : Sriamornsak, P. and Sungthongjeen, S. (2007). "Modification of theophylline release with alginate gel formed in hard capsules." **AAPS PharmSciTech** 8, 3: 1-8.

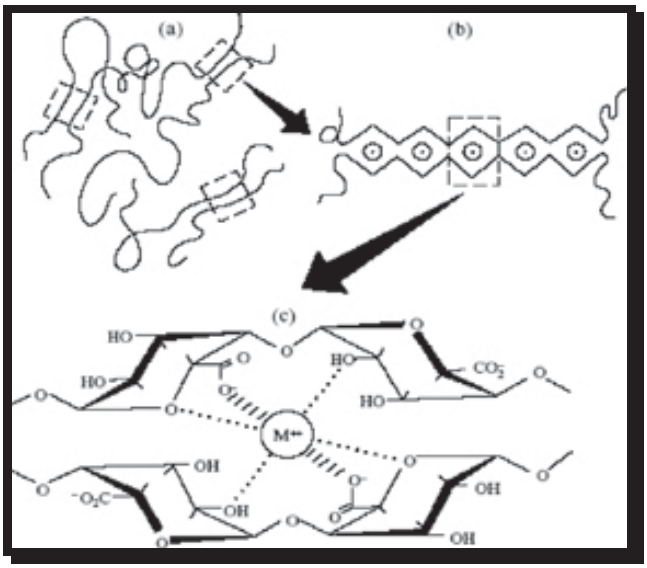

Figure 5 Schematic representation of the "egg-box" model of alginate gel. (a, b) Binding zones between polymeric alginate molecules; (c) an elementary cell of the binding zone.

Source : Patel, G. **Algae's Functional Excipient Side.** [online]. Accessed 19 March 2012. Available from http://www.pharmaquality.com.

2.5.1.2 Chitosan

Chitosan is a linear copolymer polysaccharide consisting of β (1 – 4)-linked 2-amino-2-deoxy- D–glucose (D-glucosamine) and 2-acetamido-2-deoxy- D-glucose (N-acetyl-D-glucosamine) units (Figure 6).

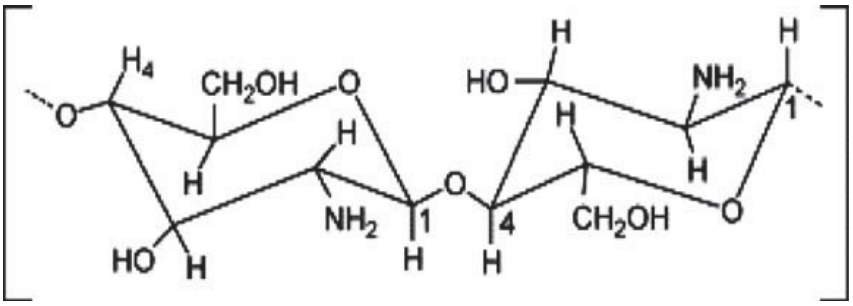

Figure 6 Chemical structure of chitosan.

Source : George, M. and Abraham, T.E. (2006). "Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan-a review." **J Control Release** 114, 1: 1–14.

The most commonly obtained form of chitosan is the α-chitosan from crustacean chitin obtained from crab- and shrimp shell wastes (Shahidi, Reader and Falshaw, 1997: 535-542; Roberts, 1992: 274-315). This accounts for approximately 70% of the organic compounds in such shells. In preparing chitosan, ground shells are deproteinated and demineralized by sequential treatment with alkali and acid, after which the extracted chitin is deacetylated to chitosan by alkaline hydrolysis at high temperature. Production of chitosan from these sources is inexpensive and easy. The term chitosan is used to describe a series of polymers of different degrees of deacetylation (DD), defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular weights (Mw) (Shahidi, Arachchi and Jeon, 1999: 37-51). The DD of typical commercial chitosan is usually between 70% and 95%, and the Mw between 10 and 1000 kDa. The properties, biodegradability and biological role of chitosan are frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues. Chitosan has been widely used in food industry and is an approved food additive in Japan. Chitosan has also been marketed throughout the world as a component in non-medical products, as a fat

binder in cholesterol lowering and slimming formulations (Kanauchi et al., 1995: 786-790). It has been claimed that chitosan entraps lipids in the intestine, because of its cationic nature (Wuolijoki, Hirvela and Ylitalo, 1999: 357-361; Mi et al., 1999: 1551-1564). It has also been studied in biomedical field and has been found to be highly biocompatible. The hydrogel preparation and drug entrapment can be done under relatively mild gelation conditions. Chitosan solution containing the desired protein can be formed into a gel upon contact with a cross-linking agent. One of the commonly used cross-linking agents for the ionic gelation of chitosan is tripolyphosphate (TPP). It is a non-toxic polyanion which can interact with chitosan via electrostatic forces to form ionic cross-linked networks. It can be used for the preparation of chitosan beads and microspheres because of its quick gelling ability (Porporatto, Bianco and Correa, 2005: 62-69).

Chitosan, a biodegradable and biocompatible polysaccharide with immunological activity (Babensee and Paranipe, 2005: 503-510; Chopra et al., 2006: 1021-1032), which acts both as a bioadhesive (Thanou, Verhoef and Junginger, 2001: 139-144) and as efficient absorption enhancer (Lubben et al., 2001: 139-144), has also been regarded as a promising polymer for the formulation of vaccine delivery systems, especially for application to mucosal surfaces (O'Hagan, 2006: 123-147). Recently, it has been designed as delivery system composed of a chitosan core to which the hepatitis B surface antigen (HBsAg) was adsorbed and was then coated with sodium alginate. This delivery system was recently evaluated for mucosal routes of immunization. On the other hand, the evaluation of chitosan as an adjuvant for parenteral vaccination has been less studied and, in most cases, the results of these vaccination studies were reported together with the results of intranasal or oral vaccination studies, making the possible value of chitosan as an adjuvant for parenteral routes less noticeable in the scientific literature. Generally speaking, the development of safe novel adjuvants is necessary not only for the more challenging environment of the mucosal surfaces, but also for parenteral vaccination, to maximize the efficacy of new or already available vaccines. In the last few years this idea became even more urgent since newer generations of antigens are predominantly purified recombinant proteins, which are often poorly immunogenic. Additionally, the new generation of adjuvants may also allow vaccination strategies to be applied to

novel areas, including “therapeutic” vaccines designed to control allergies, auto-immune diseases, malignancies, drug dependencies, neural diseases, or fertility (Peluso et al., 1994: 1215-1220). Chitosan suspensions or microparticles have also been shown to possess immune stimulating capacity, such as the accumulation (via chemotaxis) and specific activation (NO production) of macrophages (Qin et al., 2002: 111-117), suppress tumor growth (Nishimura et al., 1984: 93-99), induce non-specific resistance to infections (Iida et al., 1987: 270-274; Lee et al., 2002: 645-658), induce cytokines like IL-2, IL -10, TNF α, IL-12,IFN γ (Porporatto et al., 2004: 433-441; Seferian and Martinez, 2000: 661-668), enhance antibody responses and delayed type hypersensitivity (Shibata et al., 2001: 6123-6130; Nishimura et al., 1985: 379-384) and activate cytotoxic T-cells as well as natural killer cells (Schipper et al., 1999: 335-343). In an *in vitro* Caco-2 cell monolayer system, chitosan suspensions were able to penetrate the tight junctions and, therefore, allowed the enhanced paracellular transport of co-administered drugs across the epithelium (Schipper et al., 1997: 923-929, 1996: 1686-1692; Lubben et al., 2001: 201-207). Depending on the chitosan preparation, the model drugs atenolol or ^{14}C-mannitol were transported in 10–40 times higher a mounts across the Caco-2 cell layers compared to baseline permeability. In contrast to the soluble formulation, chitosan particles were endocytosed by the M-cells of Peyer’s patches and biodegraded by macrophages (Lubben et al., 2001: 687-694, 2002: 449-456; Bernkop-Schnurch, Guggi and Pinter, 2004: 177-186). By derivatization with different coupling reagents and thiol groups, respectively (thiolated chitosans, thiomers), the mucoadhesiveness, the paracellular transport , the in situ gelling features, the cohesion and the stability could be increased, guaranteeing a prolonged release of embedded substances (Lubben et al., 2002: 449-456; Bernkop-Schnurch, Guggi and Pinter, 2004: 177-186, 2004: 9-17). Chitosan has only limited ability for controlling the release of encapsulated compound due to its hydrophilic nature and easy solubility in acidic medium. Therefore it needs to be cross-linked for drug delivery applications. To improve the drug delivery properties of chitosan, several modifications have also been studied. By simple covalent modifications of the polymer, its physicochemical properties can be changed and can be made suitable for the peroral drug delivery purpose.

N-trimethylated chitosan (TMC) is a partially quaternized derivative of chitosan (Figure 7). It is prepared by the reductive methylation of chitosan. The degree of quaternization can be varied by varying the reaction time or the number of steps in the reaction (Bernkop-Schnurch, Guggi and Pinter, 2004: 9-17). The trimethylation of chitosan allows maintenance or improvement of the mucoadhesive properties of the starting chitosans dependent on the degree of quaternization. In particular, the mucoadhesive properties increase with increasing degree of quaternization (Cheng et al., 1998: 983-988). TMC was found to be a good absorption enhancer for peptide drugs. TMC with a degree of quaternization of 12% was able to increase the transport of several peptide drugs across Caco-2 cell mono-layers (Bernkop-Schnurch, Kast and Guggi, 2003: 95-103). The penetration enhancing activity of TMC is as well as other properties, among which is its biocompatibility. TMC improves the bioavailability of hydrophilic and large MW molecules (like peptides and proteins) when administered via buccal route (Cheng et al., 1998: 983-988). Moreover, TMC exhibits immunoadjuvants and antigen delivery systems for mucosal vaccinations (Boonyo et al., 2007: 168-175). Thus, the trimethylation helps to overcome the problems of poor solubility of chitosan in neutral and higher pH and also to improve the proper ties like mucoadhesiveness and permeation enhancing effect.

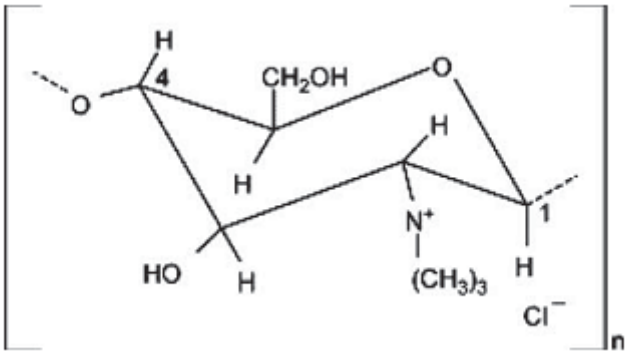

Figure 7 The chemical structure of *N*-trimethylated chitosan (TMC)

Source : Van der Merwe, S.M. et al. (2004). "Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs." **Eur J Pharm Biopharm** 58, 225-235.

2.5.1.3 Starch

Starch is one of the most important natural organic compounds, abundant in nature. It is found in the roots or fruits (Sandri et al., 2005: 146-155). The most common sources of food starch are corn, potato, wheat, tapioca and rice (Buleo, Colonna and Leloup, 1990: 515-532; Wheatley, Liping and Bofu, 1996: 70-275). Developed countries (Canada, USA, Europe and Japan) have 77% of the global starch market (Henry and Westby, 1998: 48). The food sector consumes 55% of world production versus 45% in board industries, textile, adhesive, glue and pharmaceutical products (Sansavani and Verzoni, 1998: 28). In foodstuffs, starch is used to influence or control such characteristics as, aesthetics, moisture, consistency and shelf stability. It can be used to bind, expand, densify, clarify or opacify, attract or inhibit moisture. It is also used for different textures such as stringy texture, smooth texture or pulpy texture, soft or crisp coatings, and to stabilize emulsions (DeCock, 1996: 18-22). Starch production and utilization varies a great deal between the different countries of Europe. The major crops for starch production are maize, wheat and potato. There are many potential uses of starch. Unmodified starch can be used in the pharmaceutical, paper, mining and building industries. It can be modified and converted to starch derivatives, isosugar, high fructose syrup and ethanol. Nevertheless the native starches exhibit some disadvantages certain in industrial applications. The native starch granules hydrate easily, swell rapidly, rupture, lose viscosity, and produce weak bodied, very stringy and cohesive pastes. Chemically modified starches have thus been designed to respond to industrial demand. The reticulation creates some decking among molecules, reinforcing the cohesion of the starch grain and increases its resistance to high temperature treatments (sterilization), mechanical shearing (extrusion), to acidic treatment and its stability also during freeze/thaw cycles. The stabilization by substitution of a chemical grouping (oxidation, esterification, and etherification) avoids the reorganization among the molecules after cooking. It then limits the risks of syneresis and delays or avoids the retrogradation (Amani et al., 2005: 94-101). Nowadays, consumers want to see more “natural” and “healthier” industrial products manufactured “without chemical processing” on the market. The nature of natives starches gels are resistant to high shearing, like maize and wheat,

quinoa and amaranth (Praznick et al., 1999: 197-211). Starches gels with resistance to syneresis include tapioca (Varavinit et al., 2000: 214-217), waxy maize (Yuan and Thompson, 1998: 571-573), and waxy sorghum (Howling, 1980: 51-61). The millet and quinoa's starches gels are described to be stable under acidic condition (Varavinit et al., 2000: 214-217). From all type of starches, the Dioscorea genus has a large biological diversity including more than 600 species worldwide (Hamon et al., 1997: 385-400). It has also a world with more than 37 millions tones/year. This genus appears nowadays as a source of native starches whose functional characteristics, if sufficiently exploited, could find some applications in food ingredient industry. However, publication on yam starches accounts is less than 1% of the total information available for food science and technology abstracts, and the food intelligence databases (Satin, 1998: 11). Therefore, yam starch or Dioscorea genus was focused in this review.

2.5.1.3.1 Yam starch

Yam is common name for any of several members of a genus of perennial herbs. Members of the genus are monocots and are native to tropical regions throughout the world. Yams are cultivated for their edible tubers, which grow up to 2.4 m (8 ft) long and weigh up to 45 kg (100 lb). The inconspicuous white or greenish-yellow flowers, arranged in spikes or racemes, have a six-part calyx (outer flower whorl), a six-lobed corolla (inner floral whorl), six stamens, and a solitary pistil. The fruit of the yam consists of a membranous, three-winged capsule.Yam (Dioscorea species) is a member of the mono cotyledonous family Dioscoreaceae and is a staple food in West Africa, Southeast Asia, and the Car ibbean (Akoruda, 1984: 717-733). The fresh tuber slices are widely used as functional foods in Taiwan, and the dried slices are used as traditional Chinese medicines (Liu et al., 1995: 111-126). Yams are the source of the chemical diosgenin, a precursor of progesterone, cortisone, and other medically important steroids. The collection of plants for the production of diosgenin is licensed in several parts of the world. The yam storage protein dioscorin accounts for about 90% of the extractable water- soluble proteins found in different species (D. batatas, D. alata, D. pseudojaponica) as estimated by the immunostaining method (Hou, Chen and Lin, 2000: 191-196), and dioscor in from all yam species

exhibited carbonic an hydrase and trypsin inhibitor activities (Hou, Chen and Lin, 1999b: 151-156; Reid et al., 1993: 11-159). Yam dioscorin was also found to have stimulated effects on peritoneal macrophages from BALB/c mice, but the concentrations needed to exceed 50 μg/ml. Yen-Wenn Liu et al. also studied the immunomodulatory ability of purified dioscorin from yam (Dioscorea alata L. cv. Tainong 1) tuber. The resulted showed oral administration of dioscorin in an animal model to investigate the immunomodulatory effects in vivo is currently being performed, and the preliminary results also support the view that dioscorin acts as an immunomodulatory protein.

2.5.2 Preparation of microparticles

Several techniques are available for microencapsulation, and the choice of a method depends on the physical and chemical properties of the polymer and antigen to be encapsulated, and the function and desired size of the microparticles. A high ratio of antigen to polymer is preferred to minimize the amount of mass that needs to be administered, without compromising the release kinetics. In addition, the microencapsulation technique must afford a pharmaceutically acceptable product relative to residual solvents and processing aids, batch-to-batch reproducibility, ease of scale-up, and high encapsulation efficiency and yields. For commercialization, cost-effectiveness is also an important requirement, especially for product isolation and drying and for solvent disposal. In general, the most important requirement in the development of microencapsulation techniques, especially for protein delivery is avoiding conditions that can cause adverse effect on the biological material, such as excessive heat, organic solvents, strongly acidic or basic solutions. Other critical considerations include particle size, distribution control, and microencapsulation efficiency (Table 3). Microparticles can be prepared using any of the following techniques.

Table 3 Comparison of the various processes used for the preparation of microparticles

Process used	Particle size (μm)	Polymers	Comments
Solvent evaporation	1–100	Relatively stable polymers, e.g. polyesters, polystyrene	Labile polymers may degrade during the fabrication process due to the presence of water
Solvent removal	1–300	High melting point polymers especially polyanhydrides	Avoids use of water, only organic solvents are used
Spray drying	1–10	-	Primarily for microspheres used for intestinal imaging
Ionic gelation and size extrusion	1–300	Chitosan, CMC, alginate	Used for encapsulation of live cells or allergen
Phase inversion	0.5–5.0	Polyanhydrides	Involves low polymer loss and low drug loss during fabrication process

Source : Vasir, J.K., Tambwekar, K. and Garg, S. (2003). "Bioadhesive microspheres as a controlled drug delivery system." **Int J Pharm** 255, 13–32.

2.5.2.1 Solvent extraction and solvent evaporation

The extraction-evaporation microencapsulation methods have been widely used because they can be easily set up in a laboratory and do not require any specialized equipment. This process, the polymer is first dissolved in a suitable volatile solvent, usually methylene chloride for solvent evaporation, or acetonitrile for solvent extraction. The active agent can be incorporated into the polymer solution either as an aqueous solution, to form a primary emulsion, or as a solid matrix, which forms a dispersion. In such a system, droplet formation is a dynamic process in which droplets constantly form, collide, and coalesce or redivide. The size distribution of the droplets at steady-state depends on various parameters of the experimental setup, including shear, viscosity of the two immiscible phases, interfacial tension between the liquids, and the presence of stabilizer.

In the solvent extraction process, the solvent for polymer is dissolved away when the emulsion (or dispersion) is added to a suspension medium that is a nonsolvent for the polymer (e.g., heptanes). This leads to the formation of solid microparticles in a short period, the microparticles can be recovered either by filtration or centrifugation. Solvent extraction has been used for encapsulation of various peptides and proteins (Sato et al., 1988: 21-30). On the other hand, in the solvent evaporation process, droplet solidification occurs by evaporation of the volatile solvent at the continuous-phase-air-phase interface. Most commonly, the primary water-in-oil (w/o) emulsion is formed by the aqueous solution of the antigen in the polymer solution, which is later emulsifiled into a large volume of aqueous phase (typically, aqueous solution of a suitable emulsifier, such as polyvinyl alcohol) to form an w/o/w emulsion. In general, longer-processing times are required to obtain solid microspheres by solvent evaporation. The mixing rate and evaporation time need to be carefully controlled for reproducibility. Figure 8 shows a schematic diagram of the various steps involved in microsphere manufacture using either the solvent extraction or solvent evaporation process.

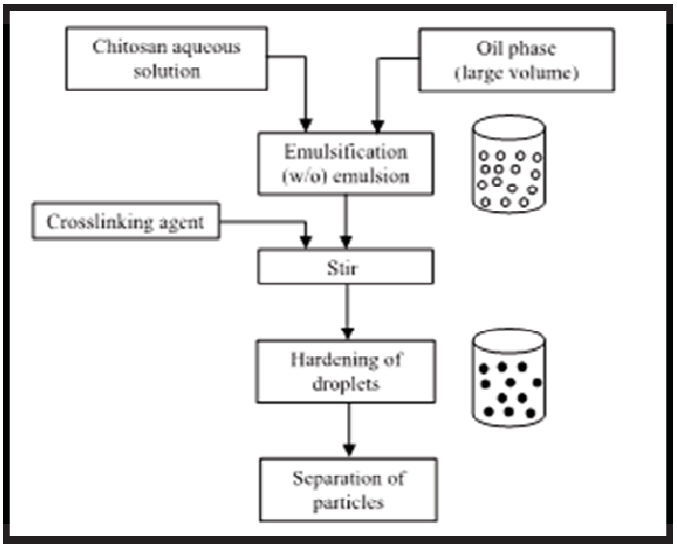

Figure 8 Schematic diagram of preparation of chitosan microparticles with emulsion cross-linking method.

Source : Agnihotri, S.A., Mallikarjuna, N.N. and Aminabhavi, T.M. (2004). "Recent advances on chitosan-based micro- and nanoparticles in drug delivery." **J Control Release** 100, 1: 5-28.

Considerable effort has been directed toward the assessment of various-processing conditions and techniques for manufacture of microparticles using these methods (Bodmeier and McGinity, 1988: 179; Cohen et al., 1991: 713). Solvent evaporation has been used to successfully encapsulate proteins, including bovine serum albumin, ovalbumin, tetanus toxoid, staphylococcal enterotoxin B toxoid, and peptides, such as leuprolide acetate (Alinso et al., 1994: 299; Eldridge et al., 1991: 2978; Ogawa et al., 1988: 1103). Entrapped volatile solvent in the microparticles can be significantly reduced by subjecting the microparticles to vacuum drying; however, trace amounts of organic solvents are often difficult to remove. To minimize the safety and regulatory concerns when dealing with organic solvents, liquid carbon dioxide under supercritical conditions has recently been used as a nonsolvent for the polymer.

2.5.2.2 Phase Separation

The earliest efforts at micromencapsulation used coacervation-phase separation techniques to produce pressure-sensitive dye microcapsules for application such as carbonless carbon paper. Gelatin and ethyl cellulose are the polymers most commonly used to make microparticles using this technique (Sampath, Garvin and Robinson, 1988: 745). Robinson et al. used a nonsolvent-induced coacervation method to encapsulate gentamicin sulfate in poly(lactide) microspheres. In this method, the polymer was dissolved in a suitable solvent (methylene chloride), to which the active drug was added to form a suspension. Hexane was used as a nonsolvent to cause separation of the polymer around the active drug particles. Once phase separation occurred completely, the microparticles were allowed to harden and then separated from the medium.

2.5.2.3 Spray drying

The spray-drying technique has not been exploited to the fullest potential for microencapsulation because of the need for specialized equipment; however, this is the easiest method to scale-up. Spray-drying converts a liquid into a powder in a single step. It involves the following four stages;

1) Atomization of feed into a spray
2) Spray-air contact
3) Drying of the spray
4) Separation of dried product

Several atomization systems are available such as rotary, pressure and pneumatic. The feed that is pumped through the atomizer, using a suitable pumping system, is either an emulsion or dispersion of the active agent in polymer solution. The feed is sprayed into the drying chamber, where air is blown in either concurrent or countercurrent pattern. Because of the availability of a large surface area for evaporation, both heat and mass transfer occur rapidly, leading to formation of dry microparticles in a very short time. The product is separated from the airstream by a cyclone separator (Bodmeier and Chem, 1988: 745). The method of microencapsulation significantly affects the characteristics of the microparticles. In general, porous, spherical particles, with a broad size distribution, that provide rapid release of incorporated active agent are obtained by the solvent extraction technique, whereas less porous microparticles are obtained by solvent extraction technique and phase separation techniques. All of these techniques provide high yields on a laboratory scale, but are difficult to scale up. Spray-drying provides porous powders that are approximately spherical with a narrow size distribution. The technique is also easily scalable; however, the microsphere yields are significantly lower on the laboratory scale. A schematic diagram of microparticles preparation with spray dry method is shown in Figure 9.

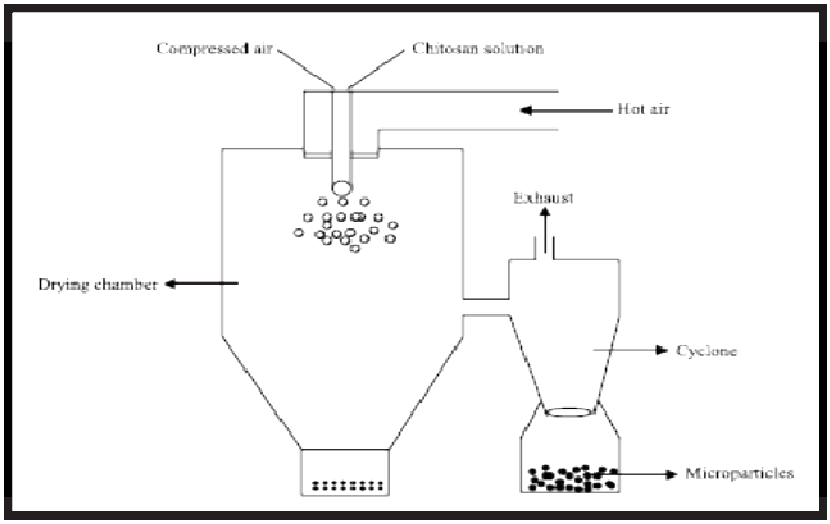

Figure 9 Schematic diagram of microparticles preparation with spray dry method.
Source : Agnihotri, S.A., Mallikarjuna, N.N. and Aminabhavi, T.M. (2004). "Recent advances on chitosan-based micro- and nanoparticles in drug delivery." **J Control Release** 100, 1: 5-28.

2.5.2.4 Ionic gelation

This method is based on the conjugation of oppositely charged macromolecules for preparing microparticle. Microparticles made of gel-type polymers, such as alginate, are produced by dissolving the polymer in an aqueous solution, suspending the active ingredient in the mixture and extruding through a precision device, producing microdroplets which fall into a hardening bath that is slowly stirred. The hardening bath usually contains calcium chloride solution, whereby the divalent calcium ions crosslink the polymer forming gelled microspheres controlled by using various size extruders or by varying the polymer solution flow rates. Dambies et al. prepared chitosan gel beads using molybdate as the gelling agent (Dambies et al., 2001: 1198-1205). It was observed that this new gelation technique led to a structure different from one produced during alkaline coagulation of a chitosan solution. Instead of a morphology characterized by large open pores, gel beads produced in a molybdate solution, under optimum conditions (pH 6; molybdate concentration, 7 g/l), were found to have a double layer structure corresponding to a very compact 100 μm thick external layer and an internal structure of small pores.

The method involves an “all-aqueous” system and avoids residual solvents in microspheres. They have some studied developed this method for encapsulation of live cells, as it does not involve harsh conditions, which could kill the cells. The surface of these microspheres can be further modified by coating them with polycationic polymers, like polylysine after fabrication. The particle size of microspheres can be controlled by using various size extruders or by varying the polymer solution flow rates. Figure 10 shows a schematic diagram of preparation of chitosan microparticles with ionic gelation method.

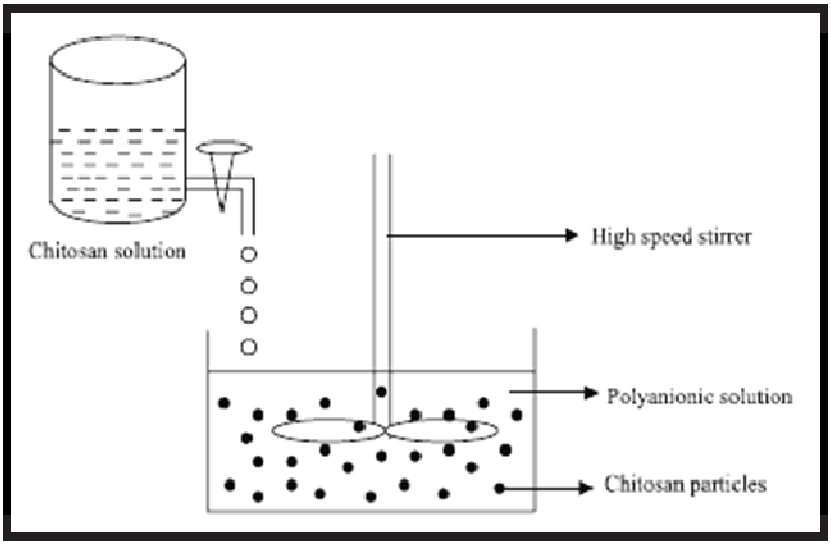

Figure 10 Schematic diagram of preparation of chitosan microparticles with ionic gelation method.

Source : Agnihotri, S.A., Mallikarjuna, N.N. and Aminabhavi, T.M. (2004). “Recent advances on chitosan-based micro- and nanoparticles in drug delivery.” **J Control Release** 100, 1: 5-28.

2.5.3 Evaluation of the bioadhesive microparticles

The best approach to evaluate bioadhesive microparticles is to evaluate the effectiveness of mucoadhesive polymer to prolong the residence time of drug at the site of absorption, thereby increasing absorption and bioavailability of the drug. The methods used to evaluate bioadhesive microsparticles include the following;

2.5.3.1 Wilhelmy plate technique

The Wilhelmy plate technique is traditionally used for the measurement of dynamic contact angles and involves the use of a microtensiometer or a microbalance. The microbalance unit consists of stationary sample and tare loops and a motor powered translation stage. The instrument measures the bioadhesive force between mucosal tissue and a single microsphere mounted on a small diameter metal wire suspended from the sample loop in microtensiometer An apparatus to determine mucoadhesion using Wilhemy's technique is shown is Figure 11.

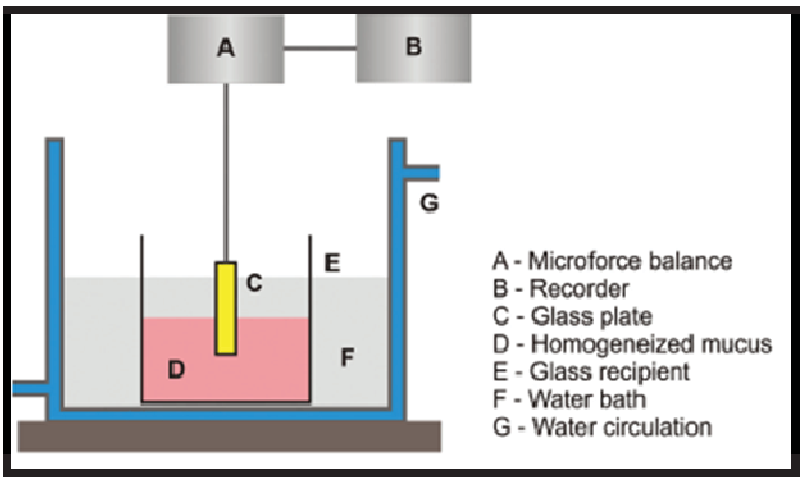

Figure 11 Apparatus to determine mucoadhesion using Wilhemy's technique.
Source : Carvalho F.C. et al. (2010). "Mucoadhesive drug delivery systems." **Braz J Pharm Sci** 46, 1: 2-17.

The tissue, usually rat jejunum, is mounted within the tissue chamber containing Dulbecco's phosphate buffered saline containing 100 mg/dl glucose and maintained at the physiologic temperature. The chamber rests on a mobile platform, which is raised until the tissue comes in contact with the suspended microsphere. The contact is held for 7 min, at which time the mobile stage is lowered and the resulting force of adhesion between the polymer and mucosal tissue is recorded as a plot of the load on microsphere versus mobile stage distance or deformation. The plot of output of the instrument is unique in that it displays both the compressive and the tensile portions of the experiment. By using the CAHN software system, three essential

bioadhesive parameters can be analyzed. These include the fracture strength, deformation to failure and work of adhesion (Santos et al., 1999: 113-122).

2.5.3.2 Falling liquid film method

It is a simple, quantitative *in situ* method, wherein an excised intestinal segment cut lengthwise, is spread on a plastic flute and positioned at an incline. The suspension of microspheres is allowed to flow down the intestinal strip. Particle concentrations entering the segment from the dilute suspension reservoir and leaving the intestinal segment can be determined with the help of Coulter counter to quantify the steady state fraction of particles adhered to the intestinal mucosa. The percent of particles retained on the tissue is calculated as an index of bioadhesion. A schematic of falling liquid film method is shown in Figure 12 (Teng and Ho 1987: 133-149).

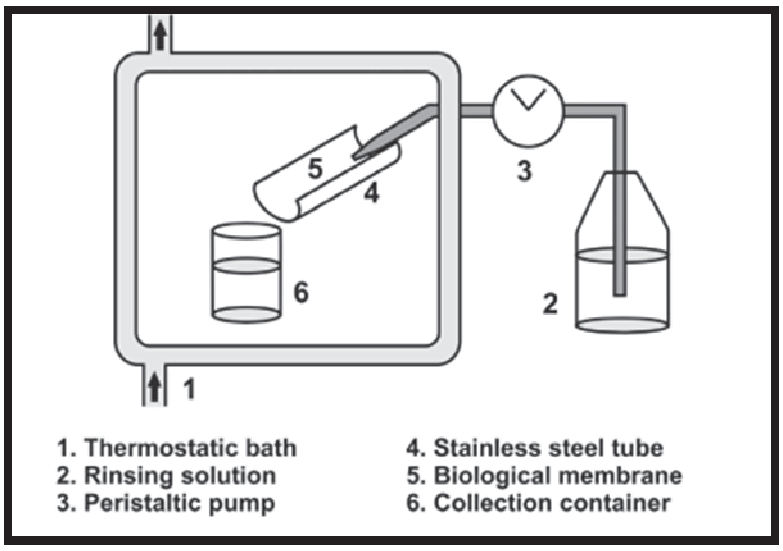

Figure 12 Schematic of falling liquid film method.

Source : Carvalho F.C. et al. (2010). "Mucoadhesive drug delivery systems." **Braz J Pharm Sci** 46, 1: 2-17.

2.5.3.3 Everted sac technique

The everted intestinal sac technique is a passive test for bioadhesion involves polymeric microspheres and a section of the everted intestinal tissue. It is performed using a segment of intestinal tissue excised from the rat, everted, ligated at

the ends and filled with saline. It is then introduced into a tube containing a known amount of the microspheres and saline, and agitated while incubating for 30 min. Sac is then removed, microspheres are washed and lyophilized, and the percentage of binding to the sac is calculated from difference in the weight of the residual spheres from the original weight of the microspheres. The advantage of the technique is that no external force is applied to the microspheres being tested; microspheres are freely suspended in buffer solution and made to come in contact with the everted intestinal tissue randomly. The CAHN technique (4.3.1) and the everted intestinal sac technique, both predict the strength of bioadhesion in a very similar manner (Santos et al., 1999: 113-122).

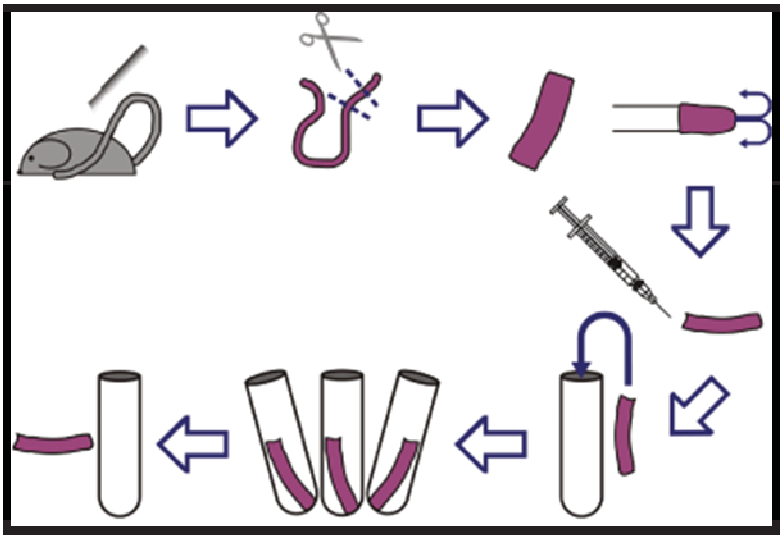

Figure 13 Everted gut sac procedure.

Source : Carvalho F. C. et al. (2010). “Mucoadhesive drug delivery systems.” **Braz J Pharm Sci** 46, 1: 2-17.

2.6 Antibody titer

An antibody titer is a measurement of how much antibody an organism has produced that recognizes a particular epitope, expressed as the greatest dilution that still gives a positive result. Enzyme-linked immunosorbent assays (ELISA) is a common means of determining antibody titers. ELISA is a widely-used method for measuring the concentration of a particular molecule such as peptides, proteins,

antibodies and hormones in a fluid such as serum or urine. It is also known as enzyme immunoassay or EIA**.** In ELISA a liquid sample is added onto a stationary solid phase with special binding properties and is followed by multiple liquid reagents that are sequentially added, incubated and washed followed by some optical change (e.g. color development by the product of an enzymatic reaction) in the final liquid in the well from which the quantity of the analyte is measured. The qualitative reading usually based on detection of intensity of transmitted light by spectrophotometric which involves quantitation of transmission of some specific type of light through the liquid. The sensitivity of detection depends on amplification of the signal during the analytic reactions. Since enzyme reactions are very well known amplification processes, the signal is generated by enzymes which are linked to the detection reagents in fixed proportions to allow accurate quantification thus the name "Enzyme linked". The antigen is then detected either directly (labeled primary antibody) or indirectly (labeled secondary antibody). The most powerful ELISA assay format is the sandwich assay. This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody. The sandwich format is used because it is sensitive and robust. An ELISA can also be performed as a competitive assay. This is common when the antigen is small and has only one epitope, or antibody binding site. One variation of this method consists of labeling purified antigen instead of the antibody. Unlabeled antigen from samples and the labeled antigen compete for binding to the capture antibody. A decrease in signal from purified antigen indicates the presence of the antigen in samples when compared to assay wells with labeled antigen alone.

2.6.1 Direct ELISA

The direct detection method uses a labeled primary antibody that reacts directly with the antigen. Direct detection can be performed with antigen that is directly immobilized on the assay plate or with the capture assay format. Direct detection is not widely used in ELISA but is quite common for immunohistochemical staining of tissues and cells.

2.6.2 Indirect ELISA

The indirect detection method uses a labeled secondary antibody for detection and is the most popular format for ELISA. The secondary antibody has specificity for the primary antibody. In a sandwich ELISA, it is critical that the secondary antibody be specific for the detection primary antibody only (and not the capture antibody) or the assay will not be specific for the antigen. Generally, this is achieved by using capture and primary antibodies from different host species (e.g., mouse IgG and rabbit IgG, respectively). For sandwich assays, it is beneficial to use secondary antibodies that have been cross-adsorbed to remove any antibodies that have affinity for the capture antibody. The steps of direct and indirect of ELISA are as follows in Figure 14**.**

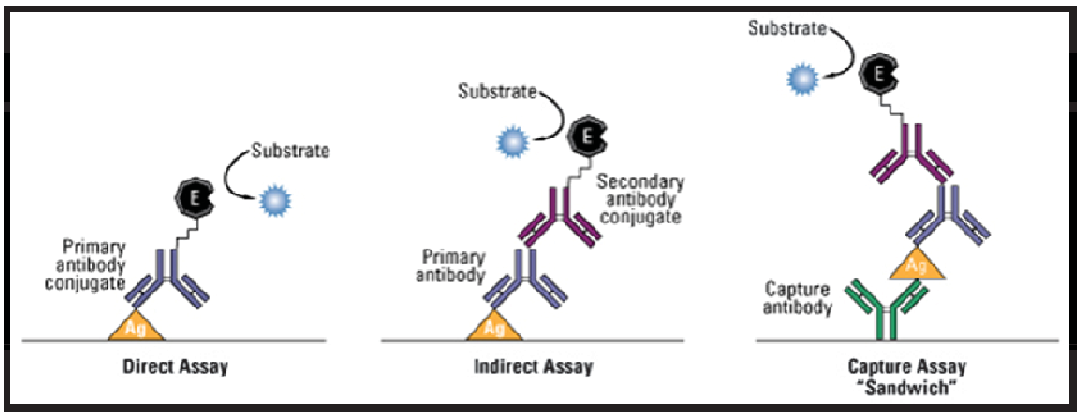

Figure 14 Common ELISA formats.

Source : Thermo Scientific. **Overview of ELISA**. [online]. Accessed 19 March 2012. Available from http://www.piercenet.com.

2.6.3 Competitive ELISA

The competitive ELISA is used to quantify antigen using competitive method. Briefly, the free antigen and antibody are incubated to form antigen-antibody complex and then the complex are bound to antigen-coated surface in the assay plate. The unbound antibody-antigen complex is washed off before adding enzyme-linked secondary antibody against the primary antibody. The substrate is then added and the antigen concentration can then be determined by the signal strength elicited by the

enzyme-substrate reaction. In this assay, the enzyme-linked secondary antibody complete with the sample antigen is associated with the primary antibody (Chaeychomsri, 2012) (Figure 15).

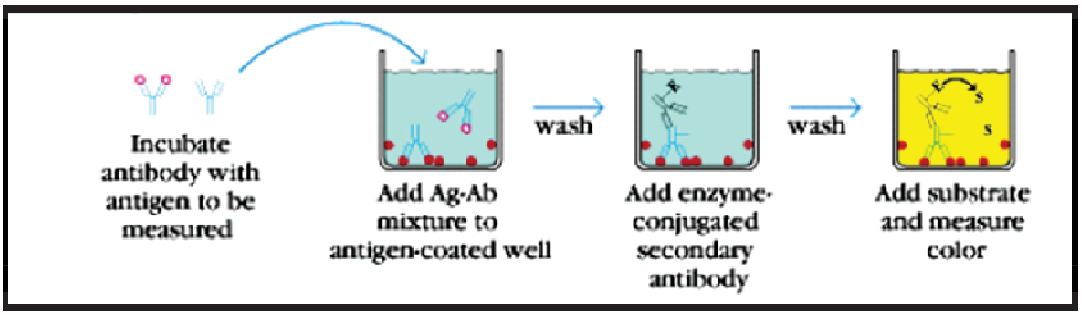

Figure 15 Competitive ELISA protocol.

Source : Chaeychomsri. **Antigen-Antibody reaction**. [online]. Accessed 19 March 2012. Available from https://pirun.ku.ac.th.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.2 Equipments

3.3 Methods

3.3.1 Preparation of microparticles

3.3.1.1 Preformulation of BSA loaded Calcium alginate microparticles (BSA-Ca-alginate)

3.3.1.2 Preparation of OVA loaded calcium alginate microparticles (OVA-Ca-alginate)

3.3.1.3 Preparation of OVA loaded calcium yam-alginate microparticles (OVA-Ca-Yam-alginate)

3.3.1.4 Preparation of chitosan coated OVA-Ca-alginate (OVA-CS-Ca-alginate) and OVA-Ca-Yam-alginate (OVA-CS-Ca-Yam-alginate)

3.3.2 Synthesis of water soluble chitosan derivatives

3.3.2.1 *N,N,N*-Trimethylammonium chitosan (TM-CS)

3.3.2.2 Synthesis of methylated *N*-(4-*N,N*-dimethylamino benzyl) chitosan (TM-Bz-CS)

3.3.2.3 Synthesis of methylated *N*-(4-*N,N*-dimethylamino cinnamyl) chitosan (TM-CM-CS)

3.3.2.4 Synthesis of methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS)

3.3.3 Characterization of microparticles

3.3.3.1 Particle size and zeta potential

3.3.3.2 Morphology

3.3.3.3 Chemical characterizations

3.3.3.4 Swelling measurement

3.3.4 Entrapment efficiency

3.3.4.1 Calcium assay

3.3.4.2 BSA or OVA loaded microparticles

3.3.5 *In vitro* release

3.3.6 Analysis of protein

3.3.6.1 Bradford assays

3.3.6.2 Lowry assay

3.3.7 *In vitro* mucoadhesion study

3.3.8 Evaluation of cytotoxicity

3.3.9 *In vivo* immunological study

3.3.9.1 Sample preparation for immunization

3.3.9.1.1 CS derivatives solution

3.3.9.1.2 microparticles

3.3.9.2 Oral immunization

3.3.9.3 Sample collection

3.3.9.4 Determination of immune responses

3.3.9.4.1 Determination of OVA-specific immunoglobulin G (IgG) antibody

3.3.9.4.2 Determination of OVA-specific immunoglobulin A (IgA) antibody

3.4 Statistical analysis

3.1 Materials

1. Albumin, from chicken egg white (Sigma-Aldrich Co., St. Louis, MO, USA)
2. Alginic acid sodium salt from brown algae, medium viscosity 40000 cps. (Sigma-Chemical Company St. Louis, MO, USA)
3. Alginic acid sodium salt from brown algae, low viscosity 250 cps. (Sigma-Chemical Company St. Louis, MO, USA)
4. Bovine serum albumin (BSA, fraction V, 69000 Da) (Sigma-Chemical Co. St. Louis, MO, USA)
5. Calcium chloride dihydrate (Merck, Germany)
6. Chitosan molecular weight (Mw) of 276 kDa (94% DD) (Seafresh Chitosan (lab) Co., Ltd, Thailand).
7. Chitosan hydrochloride molecular weight (Mw) of 200 KDa, *N,N,N*-Trimethylammonium chitosan (TM-CS), methylated *N*-(4-*N,N*-dimethylamino benzyl) chitosan (TM-Bz-CS), *N*-(4-*N,N*-dimethylamino cinnamyl) chitosan (TM-CM-CS) and methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS) were provided from Dr. Warayuth Sajomsang National Nanotechnology Center (NANOTEC)
8. Coomassie Brilliant Blue G-250 (Sigma-Chemical Company St. Louis, MO, USA)
9. Dimethyl sulfoxide (Fisher Scientific; analytical reagent grade)
10. Ethanol absolute (Scharlau® ET0016, Scharlau® Chemie SPAIN analytical reagent grade)
11. Ethylenediaminetetraacetic acid solution : EDTA (Sigma-Chemical Company St. Louis, MO, USA)
12. Hydrochloric acid (Scharlau Chemie S.A., Analytical grade, ACS)
13. Hydroxy naphthol Blue (Sigma-Chemical Company St. Louis, MO, USA)
14. Native Yam starch was provided from Laboratory of Asst. Prof. Nattawat Nattapulwat
15. Penta-sodium triphosphate (Sigma-Chemical Company St. Louis, MO, USA)

16. Sodium chloride (UNIVAR®Ajax Finechem; analytical reagent grade)

17. Sodium citrate (Ajax Finechem Australia, New Zealand)

18. Sodium hydroxide pellet BP (Ajax chemicals, New South Wales, Australia)

3.2 Equipments

1. 15 ml, 50 ml Centrifuge tubes-Sterile (Biologix research company, Thailand)

2. 96-well Cell Culture Cluster (Costar®; Corning Incorporated, USA)

3. Analytical balance (Sartorius CP224S, Sartorius CP3202S; Scientific promotion co.,ltd, Thailand)

4. Blunt needle 24, 26-gauge (TERUMO® SYRING with Needle,Thailand)

5. Cellulose Acetate Filter 0.2 μm (Sartorius AG. 37070 Goettingen, Germany)

6. Centrifuge (HERMLE Z300K ; Labnet®; Lab focus co.,ltd. Thailand)

7. Centrifuge (Biofuge stratus SORVALL; KENDRO, USA)

8. Eppendrof® tubes (Corning Incorporated,USA)

9. Fourier Transform Infrared Spectrophotometer (FT-IR, Nicolet 4700, Becthai, USA)

10. Freeze-dryer (Labconco, Freezone 2.5,USA)

11. Fusion Universal Microplate Analyzer (Model No : AOPUS01 and A153601; A Packard BioScience Company,USA)

12. High-voltage DC power supply (0-30 kV)

13. Inverted Microscope (ECLIPSE TE 2000-U; Model : T-DH Nikon® Japan)

14. Magnetic stirrer and magnetic bar (Becthai Bangkok Equipment & Chemical Co.,Ltd. Thailand)

15. Micropipette 0.1-2 μl, 2-20 μl, 20-100 μl, 100-1000 μl, 1-5 ml (masterpette ; Bio-Active Co.,Ltd.)

16. Micropipette tip (Bio-Active Co.,Ltd., Thailand)
17. Sartorius® filter set (Sartorius BORO 3. 3 Goettingen, Germany)
18. Scanning electron microscope (S-3400N, Hitachi, Japan)
19. Shaking Incubator (GFL 3031 Scientific Laboratory Supplies, UK)
20. Sonicator (Transsonic T460/H, Elma Hans Schmidbauer, Germany)
21. Syringe filter (25 mm diameter, 0.45 μm pore size, Chrom Tech Inc., USA)
22. Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK)

3.3 Methods

3.3.1 Preparation of microparticles

3.3.1.1 Preformulation of BSA loaded Calcium alginate microparticles (BSA-Ca-alginate)

The powders of alginate and $CaCl_2$ were weighed separately and further dissolved in water under magnetic stirring to prepare stock solutions of 0.5% w/v alginate low viscosity (ALV) or 1%, 2% and 3% w/v alginate medium viscosity (AMV) and $CaCl_2$ 3% w/v. To prepare alginate/BSA solutions, freshly prepared stock solutions of each compound were gently mixed to obtain a homogeneous final solution. Then, 10 ml alginate/BSA (5, 10, 20, 40, 60% w/w to polymer) solutions were extruded dropwisely through a needle placed into a glass syringe capped with a 20 gauge blunt needle (an internal diameter of needle is 0.9 mm) into a 150 ml of 3% w/v $CaCl_2$ solution. This set up applied voltage 18 kV for AMV and 20 kV for ALV and the distance between tip to collector and feeding rate was fixed at 30 cm and 1 ml h^{-1}, respectively. Microparticles were collected in $CaCl_2$ with gentle stirring for 30 min. The particles were separated from the $CaCl_2$ by centrifugation at 5862 x g for 10 min (Biofuge stratus SORVALL; KENDRO, USA) and then washed twice and resuspended in distilled water. This suspension was lyophilized in a freeze-dryer (LABCONCO, Freezone 2.5, USA) for 3 days. The microparticles were then kept in glass vials at 4 °C. Bare microparticles were also prepared in the same manner without the addition of BSA.

3.3.1.2 Preparation of OVA loaded calcium alginate microparticles (OVA-Ca-alginate)

As procedure 3.3.1.1, the best formulation of BSA-Ca-alginate was used to prepare OVA-Ca-alginate microparticles at the same manner with different concentrations of OVA (10, 20 and 40% to polymer). Bare microparticles were also prepared in the same manner without the addition of OVA.

3.3.1.3 Preparation of OVA loaded calcium yam-alginate microparticles (OVA-Ca-Yam-alginate)

OVA-Ca-Yam-alginate microparticles were prepared by electrosparying in the same procedure of Ca-alginate microparticles as described in section 3.3.1.1. The mixture of 1% w/v of alginate solution (ALV) and 0.5% w/v of yam starch solution was mixed with OVA in concentration 10, 20 and 40% w/w to polymer. Then, 10 ml Yam-alginate/OVA solutions were extruded dropwisely through a needle placed into a glass syringe capped with a 20 gauge blunt needle (an internal diameter of needle is 0.9 mm) into a 150 ml of 4% w/v $CaCl_2$ solution. This set up applied voltage according to the best condition from procedure 3.3.1.1. The microparticles were then kept in glass vials at 4 °C. Bare microparticles were also prepared in the same manner without the addition of OVA.

3.3.1.4 Preparation of chitosan coated OVA-Ca-alginate (OVA-CS-Ca-alginate) and OVA-Ca-Yam-alginate (OVA-CS-Ca-Yam-alginate)

Firstly, OVA-CS-Ca-alginate or OVA-CS-Ca-Yam-alginate microparticles were prepared in the same manner as described in section 3.3.1.2 and 3.3.1.3, respectively. After centrifugation, microparticles were resuspended in 0.1% w/v of chitosan derivatives. The mixture was stirred for 2 h to yield OVA-CS-Ca-alginate or OVA-CS-Ca-Yam-alginate microparticles. The microparticles were collected in $CaCl_2$ with gentle stirring for 30 min. The particles were separated from the $CaCl_2$ by centrifugation at 5862 x g for 10 min, then washed twice and resuspended in distilled water. This suspension was lyophilized in a freeze-dryer for 3 days. The

microparticles were then kept in glass vials at 4 °C. Bare microparticles were also prepared in the same manner without the addition of OVA.

3.3.2 Synthesis of water soluble chitosan derivatives

3.3.2.1 *N,N,N*-Trimethylammonium chitosan (TM-CS)

Chitosan (0.50 g) was regenerated by dissolving in 1% (w/v) aqueous acetic acid (100 mL). This solution was stirred for 1 h and then 2% (w/v) sodium bicarbonate in H_2O:MeOH; 40:60 v/v, (100 mL) was dropped slowly into the chitosan solution. Then, methanol was added to precipitate chitosan. The solution was stirred and the pH was adjusted to 9 by the addition of 15% (w/v) aqueous sodium hydroxide (NaOH). The regenerated chitosan was then recovered by filtration and kept while still moisture before used in the next step synthesis.

Regenerated chitosan (0.50 g), while still moisture, was dispersed in *N*-methyl pyrrolidone (NMP) (25 mL) at room temperature for 12 h. Then sodium iodide (1.5 g) and 15% (w/v) aqueous NaOH (3.0 mL) were added and stirred at 50°C for 15 min. Subsequently, iodomethane (1 mL) was added in three portions at 4 h intervals and stirred at 50°C for 12 h. The reaction mixture was clear and yellowish. To synthesize the higher degree of quaternization of TM-CS, TM-CS (0.50 g) was dispersed in NMP (25 mL) at room temperature for 12 h. Then sodium iodide (1.5 g) and 15% (w/v) aqueous NaOH (3.0 mL) were added and stirred at 50°C for 15 min. Subsequently, iodomethane (1 mL) was added in three portions at 4 h intervals and stirred at 50°C for 12 h. An additional iodomethane (1 mL) and small amount of NaOH pellet were added and the stirring was continued for 1 h.

After synthesis methylated derivatives of chitosan, the obtained compound was precipitated in 300 mL of acetone. The precipitate was dissolved in 15% (w/v) sodium chloride aqueous solution to exchange the iodide, and dialyzed with deionized water for 3 days to remove inorganic materials and then freeze-dried. The synthetic pathway of *N,N,N*-trimethyl chitosan (TM-CS) was shown in Figure 16.

3.3.2.2 Synthesis of methylated *N*-(4-*N,N*-dimethylamino benzyl) chitosan (TM-Bz-CS)

Chitosan (1.00 g, 6.11 meq/GlcN) was dissolved in 0.2 M acetic acid (pH = 4, 70 mL). The solution was diluted with ethanol (70 mL) and aromatic aldehyde, 4-*N,N*-dimethylaminobenzaldehyde (1.0-3.0 meq/GlcN), was added and stirred at room temperature for 1 h. At this point the pH of the solution was adjusted to 5 by adding 1N NaOH. Subsequently, 1.54 g of sodium cyanoborohydride, $NaCNBH_3$ (24.46 meq/GlcN), was added and stirred at room temperature for 24 h, followed by adjusting the pH to 7 with 15% (w/v) NaOH. If the solid appeared, it was soxhleted with ethanol:ether (1:1v/v) for 2 days and washed with ethanol several times followed by acetone and dried at room temperature under nitrogen atmosphere. Subsequently, the reaction mixture was dialyzed in distilled water for 3-4 days and then freeze-dried to give powder *N*-(4-*N,N*-dimethylaminobenzyl) chitosan.

Regenerated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan 0.50 g was dispersed in 25 mL of *N*-methyl pyrrolidone (NMP) for 12 h at room temperature. Then 1.5 g of sodium iodide and 5-15% (w/v) aqueous sodium hydroxide (3.0 mL) were added and stirred at 50°C for 15 min. Subsequently, 1 mL of iodomethane was added in three portions at 4 h intervals and stirred for 12 h at 50°C. The reaction mixture was clear and yellow in color. The isolation and purification of product was performed as described above 3.3.2.1. The synthetic pathway of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (TM-Bz-CS) was shown in Figure 17.

3.3.2.3 Synthesis of methylated *N*-(4-*N,N*-dimethylamino cinnamyl) chitosan (TM-CM-CS)

By using the same procedure as described above 3.3.2.2, 4-*N,N*-dimethylaminocinnamaldehyde was used instead of 4-*N,N*-dimethylamino benzaldehyde. The synthetic pathway of methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan (TM-CM-CS) was shown in Figure 18.

3.3.2.4 Synthesis of methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS)

By using the same procedure as described above 3.3.2.2, 4-*N*-pyridinecarboxaldehyde was used instead of 4-*N,N*-dimethylaminobenzaldehyde. The synthetic pathway of methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS) was shown in Figure 19.

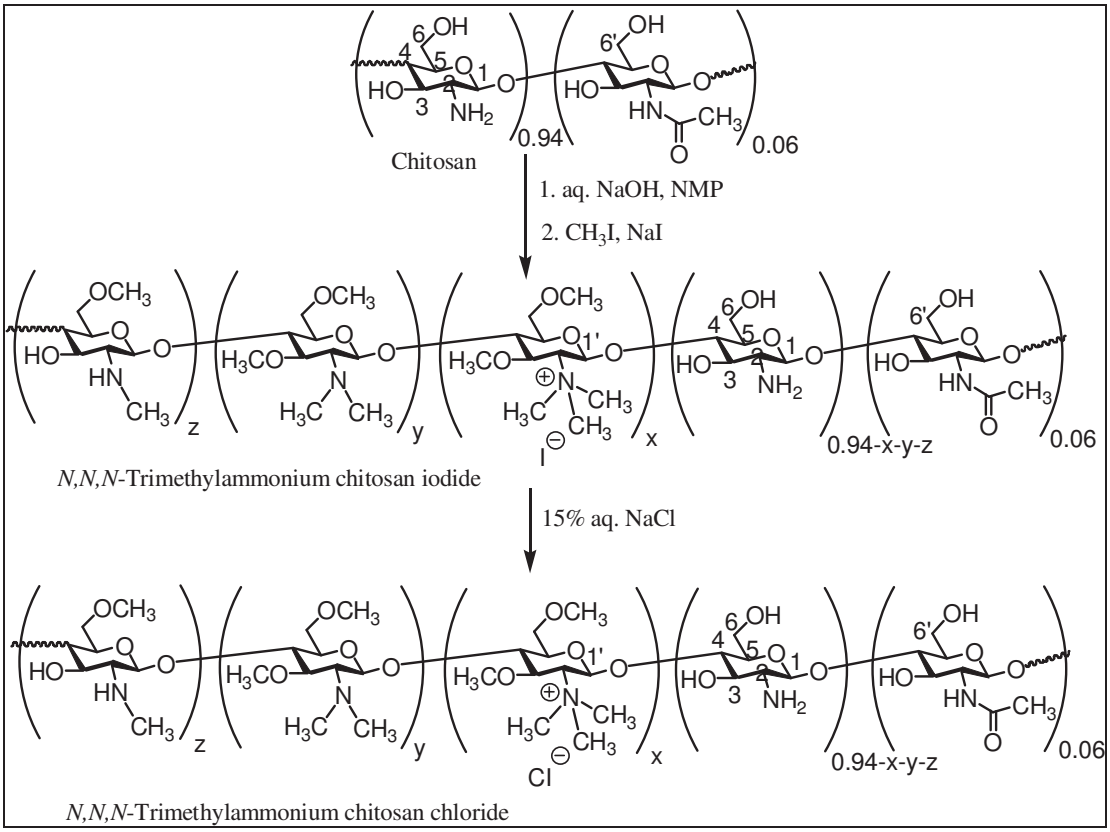

Figure 16 The synthetic pathway of *N,N,N*-trimethyl chitosan (TM-CS)

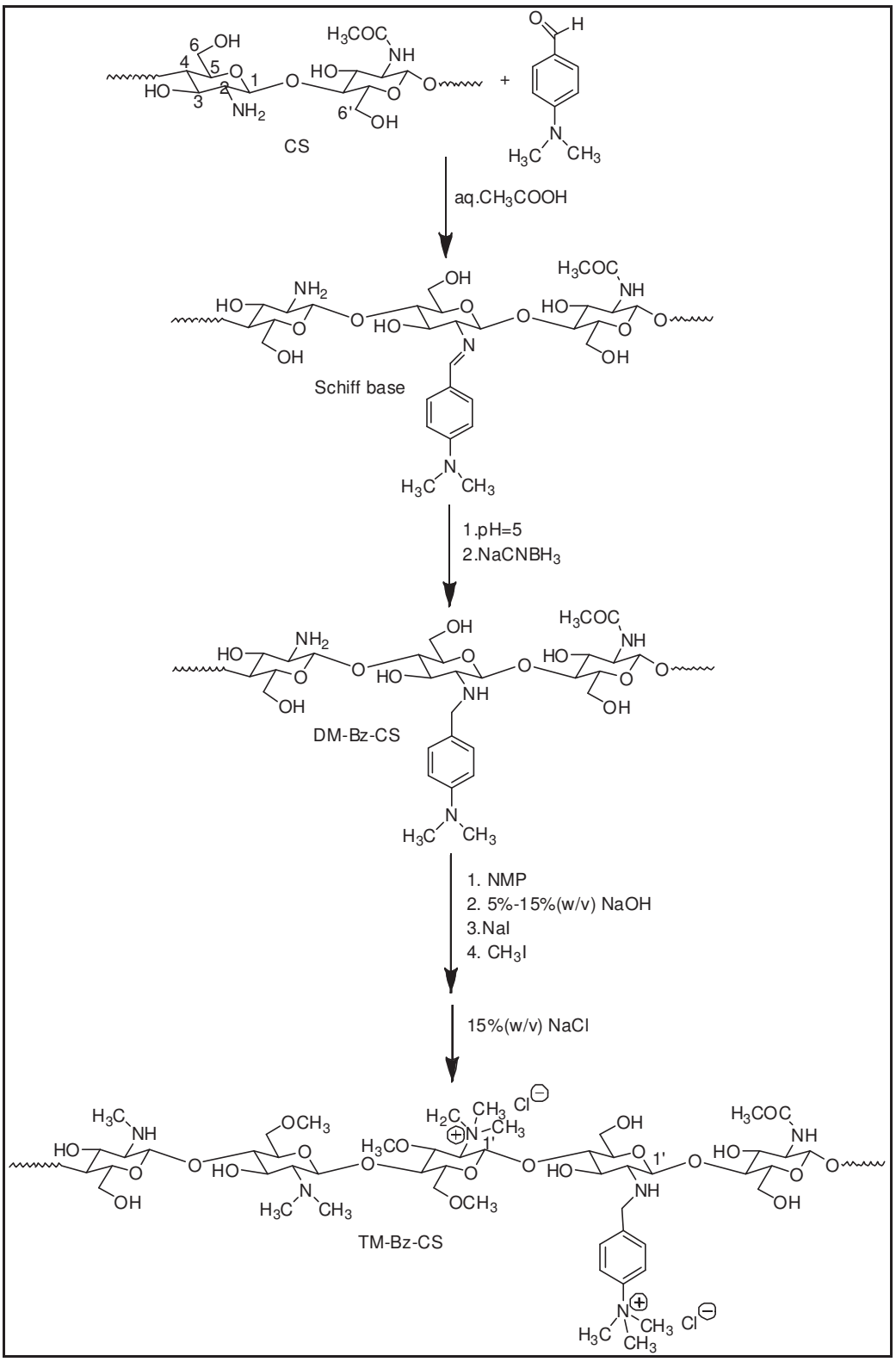

Figure 17 The synthetic pathway of methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS).

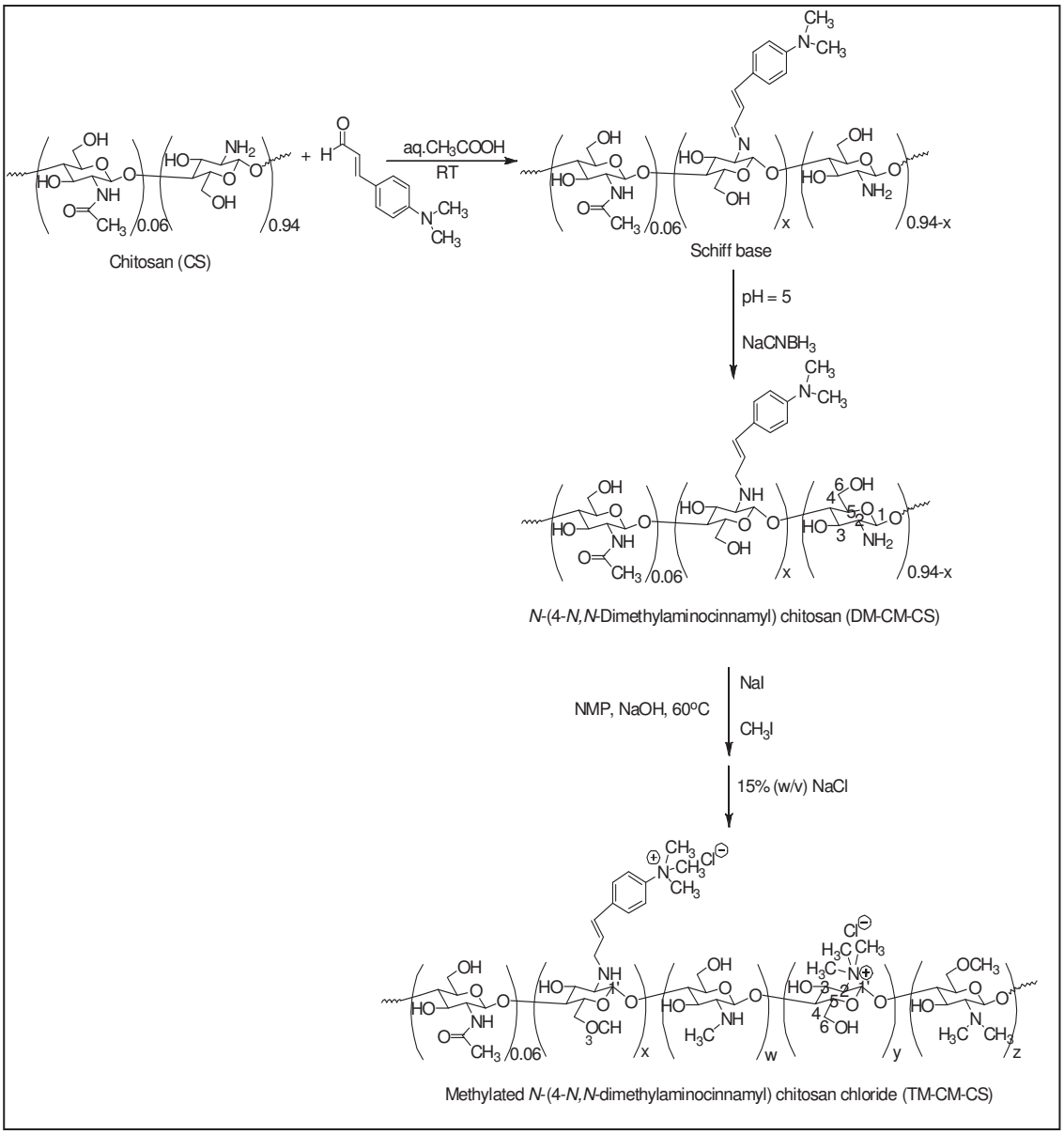

Figure 18 The synthetic pathway of methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan (TM-CM-CS).

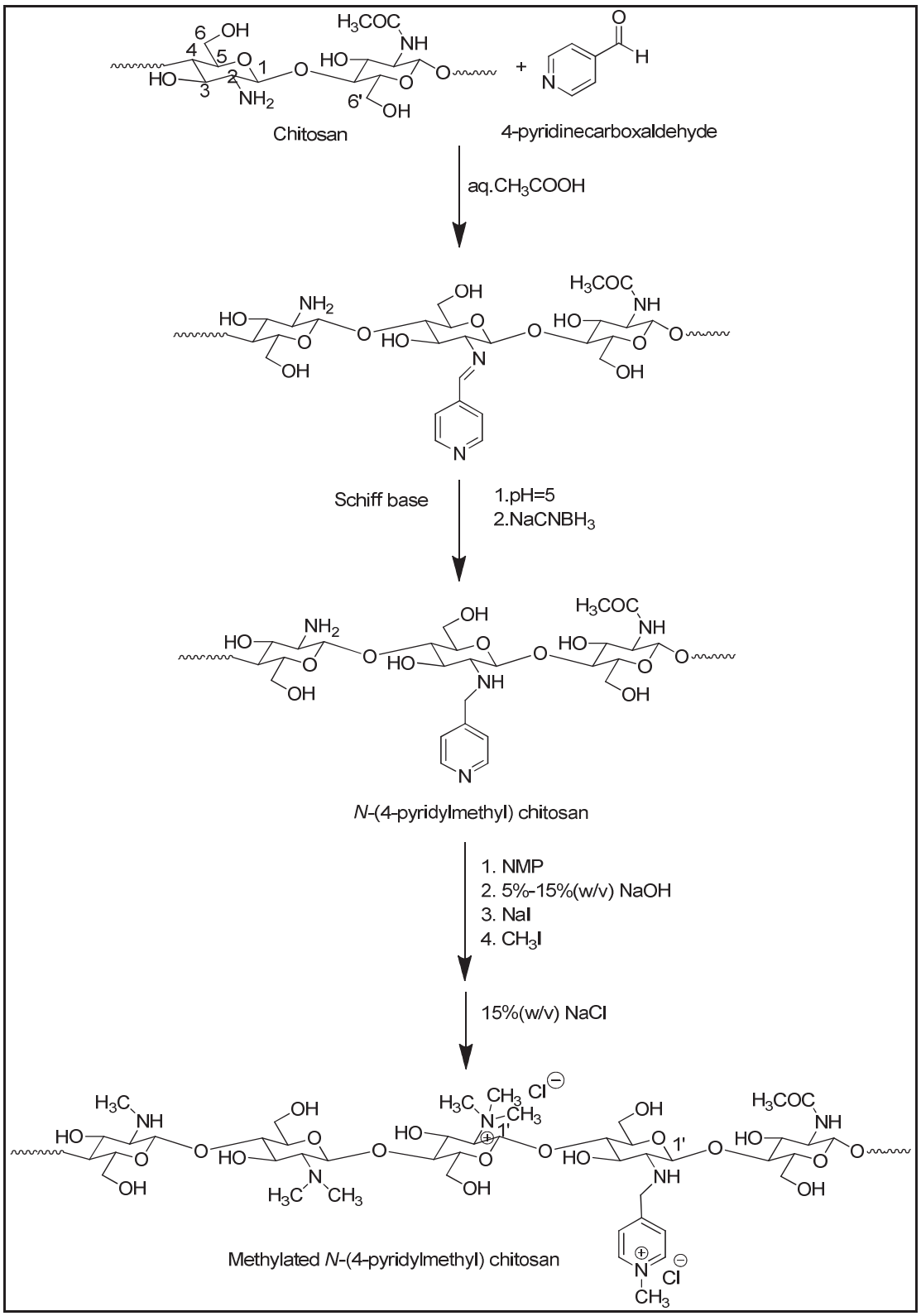

Figure 19 The synthetic pathway of methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS)

3.3.3 Characterization of microparticles

3.3.3.1 Particle size and zeta potential

The mean particle diameter and zeta potential of the protein/antigen loaded microparticles were determined in triplicate at 25°C by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The microparticle samples were diluted with distilled water that has been filtered through a 0.22 μm membrane filter.

3.3.3.2 Morphology

Morphological characterization of the microparticles was performed by inverted microscopy (Inverted Research Microscope; ECLIPSE TE 2000-U, Japan) using the magnification of 10X, 20X and by Scanning Electron Microscopy (SEM). Briefly, the microparticles were sputter coated with a thin layer of gold prior to SEM observation. The accelerating voltage was fixed at 15 kV.

3.3.3.3 Chemical characterizations

The chemical structure of the microparticles was characterized using a Fourier Transform Infrared spectrophotometer (FT-IR, Nicolet 4700, Becthai, USA) with a wave number range of 400–4000 cm^{-1}.

3.3.3.4 Swelling measurement

The swelling of microparticles was conducted in phosphate buffer solution (PBS) pH 7.4. Briefly, the sizes of dried microparticles and those after incubation in PBS for 0.5, 1, 3 and 5 h were measured by diameter of microparticles. The percentage of swelling at different time interval was determined by the difference between diameter of microsparticles at time t (D_t) and initial time (t = 0 [D_0]) as calculated from the following equation (1):

$$\text{Swelling } \% = \left((D_t - D_0)/D_0 \right) * 100 \qquad (1)$$

3.3.4 Entrapment efficiency

3.3.4.1 Calcium assay

The total content of calcium in the BSA or OVA-loaded alginate microparticles was determined by EDTA tritration as following procedure:

a) **Reagents**

Preparation of 0.05 M EDTA solution

9.3 g of EDTA was dissolved in 200 ml of distilled water and then adjusted to 500 ml in a volumetric flask.

Preparation of hydroxy naphthol blue indicator (0.1 % stock solution)

0.125 g of hydroxy naphthol blue indicator was dissolved in 40 ml of distilled water and then adjusted volume to 50 ml in a volumetric flask.

Preparation of standard 0.002 M calcium solution

Accurately weighed 0.2 g of calcium carbonate ($CaCO_3$) was dissolved in concentrated hydrochloric acid until a clear solution was obtained (no CO_2 evolution, usually 4-5 drops of conc. HCl) and then adjusted to 100 ml with distilled water in a volumetric flask.

b) Determination of molarity of standard EDTA

One hundred milliliter of calcium standard was added into 500 ml Erlenmeyer flask. After 0.25 ml of hydroxy naphthol blue indicator was added to calcium standard solution, titration was performed using EDTA solution as a titrant until wine red color turned into blue (Figure 20). The volume of EDTA was used to calculate the molarity of EDTA by equation (2). The titration was done in duplicate.

$$\text{Molarity of EDTA} = \frac{\text{mg of } CaCO_3}{(\text{MW of } CaCO_3 \times \text{ml of EDTA})} \quad (2)$$

c) Analysis of calcium in samples

Ten ml of calcium chloride ($CaCl_2$) solution before and after Ca-alginate microparticle preparation were added into 500 ml Erlenmeyer flask. To neutralize the samples, five ml of 1 M sodium hydroxide solution were added and then a few drops of hydroxy naphthol blue indicator solution were added and mixed with the samples. The solution should be in wine red color. Sample solution was titrated with standard EDTA solution with continuous swirling until the wine red color turned into blue. The volume of EDTA was used to calculate the amount of $CaCl_2$ by equation (3). The titration was done in duplicate.

$$\text{Amount of } CaCl_2 \text{ (g)/MW } CaCl_2 = \frac{\text{(molarity EDTA x ml EDTA)}}{1000} \qquad (3)$$

a) b) c)

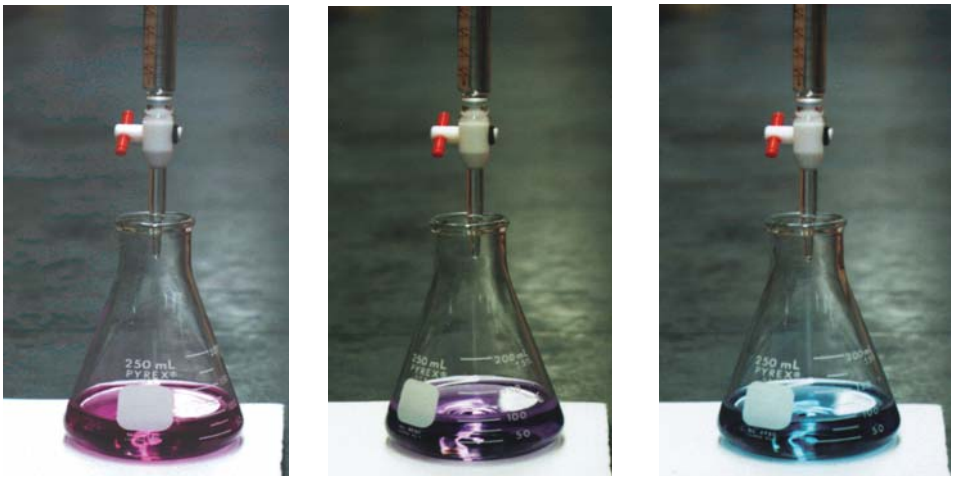

Figure 20 Analysis of calcium by titration (a) before titration (wine red color) (b) during titration with standard EDTA solution (c) at the end point

3.3.4.2 BSA or OVA loaded microparticles

The total content of BSA or OVA in the BSA or OVA-loaded alginate microparticles was determined. The dried microparticles (about 0.1 g) were accurately weighed in 15 ml centrifuge tubes containing 2 ml of 2% tri-sodium citrate buffer, followed by continuous shaking in a shaker incubator (Orbital Shaking Incubator Model: SI4) at 200 rpm until the microparticles were totally dissolved. The

supernatant was measured to determine protein concentration. The percentage of yield, percentage of entrapment efficiency and loading capacity are calculated by using equation (4)-(6), respectively:

$$\% \text{ Yield} = W_m/W_t \quad (4)$$

where W_m is the weight of microparticles, and W_t is the theoretical weight of microparticles.

$$\% \text{ Entrapment efficiency} = (P_t/ L_t) \text{ X } 100 \quad (5)$$

where P_t is the amount of BSA or OVA embedded in microparticles. L_t is the theoretical amount of BSA or OVA (obtained from feeding condition) incorporated into microparticles.

$$\text{Loading capacity} = P_t \text{ (mg)} / M_t \text{ (g)} \quad (6)$$

where P_t is the amount of BSA or OVA embedded in microparticles, and M_t is the total amount of microparticles harvested.

3.3.5 *In vitro* release

In vitro release studies of BSA or OVA-loaded microparticles were performed by suspending 10 mg of microparticles with 1 ml of PBS (pH 7.4) or simulated gastric fluid without pepsin (pH 1.2) in 1.5 ml microcentrifuge tubes. All tubes were then incubated at 37°C under shaking at 200 rpm to maintain the particles in suspension (sink conditions). To determine the amount of protein released, after a given time (1, 2, 4, 8, 12, 24, 48 h), the sample was centrifuged for 15 min at 3,000 rpm. The supernatant was measured to determine protein concentration.

3.3.6 Analysis of protein

3.3.6.1 Bradford assays

The Bradford assay was used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye,

Brilliant Blue G, and proteins in solution. The protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present.

a) Bradford reagent

50 mg of Coomassie Brilliant Blue G-250 were dissolved in 2.5 ml of 95% ethanol in 50 ml volumetric flask until completely dissolved. The solution was added to 5 ml of 85% w/v phosphoric acid, and diluted to 50 ml with water. The Bradford reagent should be light brown in color. It was stable for weeks in a dark bottle at 4°C.

b) Protein Standards

A protein standard curve was made using BSA or OVA with concentrations of 0, 12.5, 25, 50, 100 and 200 µg/ml for the standard assay of protein in microparticles.

c) Procedures

The sample was a blank, a protein standard, or an unknown sample. The blank consisted of buffer (or deionized water) with no protein. The protein standard consisted of a known concentration of protein, and the unknown sample was the solution to be assayed. 20 µl of standard protein and samples were added into 96 wells plate and then 200 µl of Bradford reagent was added and gently mixed. The mixture was assayed on absorbance at 550 nm using a microplate reader (Fusion Universal Microplate Analyzer Model: A153601).

3.3.6.2 Lowry assay

Lowry assay is a one of biochemical assay for determining the total level of protein in a solution. This method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques.

a) Lowry reagent

Reagent A:

Sodium potassium tartrate.$4H_2O$	2	g
Na_2CO_3	100	g
1N NaOH	500	ml
H_2O qs. to	1000	ml

Reagent B:

Sodium potassium tartrate.$4H_2O$	2	g
$CuSO_4.5H_2O$	1	g
1N NaOH	10	ml
H_2O	90	ml

Reagent C:

Folin's reagent : Water (1:15)

b) Protein Standards

A protein standard curve was made using BSA or OVA with concentrations of 0, 12.5, 25, 50, 100 and 200 µg/ml for the standard assay of protein in microparticles.

c) Procedures

Samples were prepared in a concentration ranging 30 to 150 µg/ml. 40 µl of each dilution of standard, samples, or buffer (for blank) were added to 36 µl of reagent A into 96 wells plate and mixed. The 96 wells plate was incubated at 50 ºC for 10 min, then cool to room temperature. 4 µl of reagent B was added to each well, mixed, and incubated plate for 10 min at room temperature. The plate was rapidly added 120 µl of reagent C to each well, mixed, and incubated the plate at 50 ºC for 10 min, then cool to room temperature. The mixture was assayed by measuring absorbance at 550 nm using a microplate reader (Fusion Universal Microplate Analyzer Model: A153601).

3.3.7 *In vitro* mucoadhesion study

The *in vitro* mucoadhesive test was carried out using a small intestine of porcine. Five centimeter segments of jejunum were everted using a glass rod. Ligatures were placed at both ends of the segment. One hundred milligrams of microparticles were scattered uniformly on the everted sac. Then, the sac was suspended in a 50 ml centrifuge tube containing 20 ml of PBS (pH 7.4). The sacs were incubated at 37 ºC and agitated horizontally. The sacs were taken out of the medium after immersion for 0.5, 1, 1.5, 2, and 2.5 h, immediately repositioned as before in a similar tube containing 20 ml of fresh PBS and dried unbound microparticles were weighed. The adhering percentage was calculated by the following equation (7):

$$\% \text{ Adhering} = 100 - \left(\frac{Wo - Wt}{Wo} \right) \times 100 \qquad (7)$$

where Wo is the weight of microparticles at initial time and Wt is the weight of unbound microparticles at the time t after incubation.

3.3.8 Evaluation of cytotoxicity

The cytotoxicity of microparticles were investigated with an MTT cytotoxicity assay. The cells were seeded at a density $2x10^4$ cells/well in 96 wells cell culture plate and pre-incubated for 24 h before microparticles treatment. The cells were treated with microparticles at various concentrations (0-20 mg/ml) in serum-free medium with pH 7.4 for 24 h. After treatment, the microparticles were removed, and fresh cell culture medium was added and incubated for 4 h to stabilize the cells. Finally, the cells were incubated with 100 µl MTT containing medium (0.1 mg/ml MTT in serum-free medium) for 4 h. Then the medium was removed, and the formazan crystal formed in living cells was dissolved in 100 µl DMSO per well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily

defined as 100%. The relative cell viability (%) was calculated according to the following equation (8):

$$\text{Relative cell viability} = \frac{(OD_{550sample} - OD_{550blank})}{(OD_{550control} - OD_{550blank})} \times 100 \quad (8)$$

where the OD_{550} (sample) is the absorbance from the wells treated with microparticles, the OD_{550} (control) is the absorbance from the wells untreated with microparticles and the OD_{550} (blank) means the absorbance of DMSO.

3.3.9 *In vivo* immunological study

3.3.9.1 Sample preparation for immunization

3.3.9.1.1 CS derivatives solution

For oral formulation, on the day of the experiments, stock solution of OVA (2 mg/ml in PBS) and CS derivatives (0.2%w/v) were mixed in ratio 1:1 to obtain a final concentration 1 mg/ml of OVA and 0.1%w/v of CS derivatives. OVA solution without CS derivatives was used for negative control.

For subcutaneous formulation (positive control), OVA solution was mixed with alum suspension which has been autoclaved to obtain a final concentration 0.5 mg/ml of OVA and 1 mg/ml of alum in PBS pH 7.4.

3.3.9.1.2 microparticles

Ca-alginate, Ca-Yam-alginate microparticles and CS derivatives coated microparticles were used for oral immunization. The microparticles containing OVA 250 µg were resuspended in 500 µl PBS pH 7.4.

3.3.9.2 Oral immunization

Female BALB/c mice, 6–8 weeks of age at the beginning of the experiment, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed at controlled temperature with free access to rodent chow and water. All studies were evaluated and performed in accordance with the Animal Ethics Committee for the use of laboratory animals, Faculty of Pharmacy, Silpakorn University, Thailand (0002/2554). The mice were

immunized with the various formulations. Mice in all groups were immunized on days 0 and 14. The mice received orally of microparticles containing 250 µg of OVA in 500 µl of PBS pH 7.4. For the positive control group, the mice were immunized subcutaneously (s.c.) in the neck region with 200 µl of alum containing 100 µg of OVA.

3.3.9.3 Sample collection

On day 0, blood samples (0.2 ml per animal) were collected from the cut tail tip. However, at the end of the study (day 21), the blood (0.6–1 ml per animal) was collected by cardiac puncture following anesthesia of the mice with diethyl ether. The blood samples were allowed to clot overnight and then centrifuged at 8,000 g for 5 min at room temperature. For tail bleeds, serum was collected and pooled for each group of mice (n = 2). For blood collected by cardiac puncture, serum from each mouse was kept separately. All serum samples were stored at -20°C until assayed.

To collect fecal samples, mice were kept separately. Freshly fecal samples (ca. 6–8 pieces per animal) were collected at the same time as blood samples. The fecal samples were kept at – 20 °C until assayed.

3.3.9.4 Determination of immune responses

Immune responses to OVA dissolved in various formulations were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) in order to determine the levels of OVA-specific serum immunoglobulin G (IgG) antibody and OVA-specific serum immunoglobulin A (IgA) as described by Pitaksuteepong (Boonyo et al. 2007: 168 – 175).

3.3.9.4.1 Determination of OVA-specific immunoglobulin G (IgG) antibody

The serum samples were assayed for sIgG by ELISA using 96 well MaxiSorp NUNC-Immuno™ plate, flat bottom, and absorbance was measured at wavelength 450 nm using a microplate reader. Briefly, 96 well MaxiSorp NUNC-Immuno™ plates, flat bottom (Nunc, Denmark) were coated with 50 µl/well of 100 µg/ml OVA in coating solution (0.1 M $NaHCO_3$, pH 8.2). After overnight incubation at 4 °C, the plates were washed 6 times with 0.05% v/v Tween 20 in phosphate buffer

saline solution (T20/PBS). Blocking was carried out by adding 200 µl of 10% v/v FBS in PBS (10FBS/PBS) into the wells followed by 2 h incubation at room temperature. The plates were then washed 6 times with T20/PBS. 100 µl of serum were added to each well in duplicate. Two fold serial dilutions of samples with 10FBS/PBS were carried out in the ELISA plates (ranging from 25 to 0.195 ng/ ml, n = 2). Blanks were also set up in duplicate using 100 µl of 10FBS/PBS and the absorbance of these blanks was subtracted from the absorbance of the standards and samples. The ELISA plates were incubated for 1 h at room temperature and then washed 6 times with T20/PBS. Goat anti-mouse IgG HRP conjugate was diluted 1:2000 with 10FBS/PBS, and 100 µl of the diluted solution were added into each well and were further incubated for 45 min at room temperature. The plates were washed 6 times with T20/PBS, and 100 µl of TMB were then added into each well for 10 min. Color development was stopped by adding 100 µl/well of 1 N H_2SO_4. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA).

3.3.9.4.2 Determination of OVA-specific immunoglobulin A (IgA) antibody

Following freeze drying of frozen fecal samples using a Freeze-dryer (LABCONCO, Freezone 2.5,USA), secretory IgA was extracted from the fecal samples by adding PBS at a ratio of 15 µl/mg dry feces and the samples were then extensively vortexed. Subsequently, the suspensions were centrifuged at 12,000 g for 20 min. The clear supernatants were assayed for sIgA by ELISA using 96 well MaxiSorp NUNC-Immuno™ plate, flat bottom. Briefly, the plates were coated with 50 µl/well of 250 ng/ml IgA kappa and 100 µg/ml of OVA solution for standard and sample wells, respectively. For standard wells, doubling dilution in the ELISA plate was performed to obtain concentration of IgA kappa ranging from 250 to 3.9 ng/ml. Blanks were also set up in duplicate using 100 µl of 10FBS/PBS. Following overnight incubation at 4 °C, the plates were washed 6 times with T20/PBS. Non-specific protein-binding sites were blocked by adding 200 µl of 10FBS/PBS into the wells followed by 2 h incubation at room temperature. The plates were then washed 6 times

with T20/PBS. One hundred microliters of sample were added to each of the sample well in duplicate, while 100 μl/well of 10FBS/PBS was added into the wells set as standard and blank. The ELISA plates were incubated for 2 h at room temperature and then washed 6 times with T20/PBS. Goat anti-mouse IgA was diluted 1:2000 with 10FBS/PBS and 100 μl of the diluted solution were added into each well and further incubated for 30 min at room temperature. Then, 100 μl of rabbit anti-goat IgG peroxidase conjugate 1:2000 with 10FBS/PBS was added into each well. After 15 min incubation at room temperature, the plates were washed 6 times. Subsequently, 100 μl of TMB was added into each well and the color was allowed to develop about 10 min. The reaction was stopped by adding 100 μl/well of 1 N H_2SO_4, and absorbance was measured at wavelength 450 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA).

3.4 Statistical analysis

All experimental measurements were performed in triplicate. Result values were expressed as mean value ± standard deviation (S.D.). Statistical significance of differences was examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test. The significance level was set at $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preparation of microparticles

- 4.1.1 Preformulation of BSA loaded Calcium alginate microparticles (BSA-Ca-alginate)
  - 4.1.1.1 Preparation of Ca-alginate microparticles
  - 4.1.1.2 BSA entrapment efficiency
  - 4.1.1.3 *In vitro* BSA release
- 4.1.2 Ovalbumin (OVA) loaded calcium alginate microparticles (OVA-Ca-alginate)
- 4.1.3 OVA loaded calcium yam-alginate microparticles (OVA-Ca-Yam-alginate)
- 4.1.4 Chitosan coated Ca-alginate microparticles (CS-Ca-alginate)
- 4.1.5 Water soluble chitosan derivatives coated OVA-Ca-alginate and OVA-Ca-Yam-alginate
  - 4.1.5.1 Synthesis of chitosan derivatives
  - 4.1.5.2 Cytotoxicity of chitosan derivatives
  - 4.1.5.3 Adjuvant activity of chitosan derivatives
    - 4.1.5.3.1 OVA-specific immunoglobulin G (IgG) antibody
    - 4.1.5.3.2 OVA-specific immunoglobulin A (IgA) antibody

4.2 Characterization of OVA loaded microparticles

- 4.2.1 Particle size and zeta potential
- 4.2.2 Morphology
- 4.2.3 Chemical characterization
- 4.2.4 Swelling property
- 4.2.5 *In vitro* mucoadhesion study

4.1 Preparation of microparticles

4.1.1 Preformulation of BSA loaded Calcium alginate microparticles (BSA-Ca-alginate)

4.1.1.1 Preparation of Ca-alginate microparticles

Alginate beads, produced in all-aqueous media by cross-linking sodium alginate with divalent cations, are widely used for the encapsulation of drugs, proteins, cells and oligonucleotides. The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations and the stacking of these guluronic groups to form the characteristic egg-box structure (Gombotz and Wee, 1998: 267-285; Lambert et al., 2001: 99-112). The simplest method of producing Ca^{2+}-alginate beads is based on dropwise sodium alginate in $CaCl_2$, using a syringe with a needle or a pipette (Elqin, 1995: 1157-1161). The diameter of the beads formed is dependent on the size of the needle used and the viscosity of the alginate solution. A larger diameter needle and higher viscosity solutions will produce larger diameter beads. The viscosity of sodium alginate can also influence the shape of the beads produced. The beads become more spherical as the concentration of the sodium alginate solution is increased. However, in general, sodium alginate solutions of greater than 5% (w/v) are difficult to prepare. An alternative consists of the dropwise addition of calcium chloride into the sodium alginate solution under high speed stirring. In the case of alginate microbeads or microparticles preparation, there are three widely known methods, namely atomization, emulsification and coacervation. The most commonly used technique is an atomization or spraying method using an extrusion device with a small orifice (Bowersock et al., 1996: 209-220; Gombotz and Wee, 1998: 267-285). The sizes of these microparticles can be controlled by either the pressure of the infusing nitrogen gas, the flow-rate of the syringe pump or the distance between the orifice and the surface of the crosslinking solution. Fine droplets of sodium alginate and protein solution will form the microparticles when cross-linked with the divalent solution. Complex coacervation of oppositely charged polyelectrolytes has also been extensively used to prepare and strengthen alginate microparticles.

Recently, electrospinning and electrospray have been developed for fabrication of fibres and particles for various applications. The electrospray or electrohydrodynamic atomization (EHDA) technique was widely employed to fabricate inorganic nanoparticles, thin films, fibres, hollow fibres, deposition of nanoparticle clusters,-micro/nanoencapsulation and production of pharmaceutical particles (Ijsebaert et al., 2001: 2735–2741). Ding et al. (2005: 395–413) reported an EHDA technique to fabricate uniform microparticles in the range of 1–15 mm. Xie et al. (2006: 3321–3332) fabricated biodegradable polymeric particles by EHDA for applications in sustained delivery of paclitaxel to treat C6 glioma in vitro. Therefore, this study focused on using the EHDA technique to continuously generate the fine droplets of sodium alginate, and protein solution will form the microparticles when cross-linked with the divalent solution.

To investigate the conditions for microparticles formation, systems able to produce microparticles were identified i.e. molecular weight of alginate, the concentration of alginate and the concentration of calcium chloride and voltage applied. To determine concentration limit in microparticle formation of alginate in the presence of divalent cations, several systems with decreasing alginate and calcium chloride concentrations in different applied voltage were prepared. Table 4 and 5 show the final composition of each system and its corresponding macroscopic evaluation and the morphology of calcium alginate microparticles in different formula.

Table 4 Macroscopic evaluation of calcium alginate particles formulation

Na alginate (%w/v)	$CaCl_2$ (%w/v)	Alginate:$CaCl_2$ (w/w)	Applied voltage (kV)	Macroscopic evaluation of alginate particles
3*	2	1.5 : 1	10–17	Large sphere particles
2*	2	1 : 1	10–12	Large sphere particles
2*	2	1 : 1	13–17	Large tail particles
2*	1	2 : 1	10–12	Large sphere particles
2*	1	2 : 1	13–17	Large tail particles
1*	0.1	10 : 1	10–17	Long rod gel beads
1*	0.25	4 : 1	10–17	Small amorphous form
1*	0.5	2 : 1	10–17	Small amorphous form
1*	1	1 : 1	10–17	Small amorphous form
1*	1.5	0.67 : 1	10–14	Small semi-circle particles
1*	1.5	0.67 : 1	15–17	Small tail particles
1*	2	0.5 : 1	10–17	Small semi-circle particles
1*	2.5	0.4 : 1	10–14	Small sphere particles
1*	2.5	0.4 : 1	15–17	Small tail particles
1*	3	0.33 : 1	10–17	Small sphere particles
0.5**	1	0.5 : 1	10–17	Small amorphous form
0.5**	1.5	0.33 : 1	10–16	Small semi-circle particles
0.5**	1.5	0.33 : 1	17–20	Small semi-circle particles
0.5**	2	0.25 : 1	10–16	Small semi-circle particles
0.5**	2	0.25 : 1	17–20	Small semi-circle particles
0.5**	2.5	0.2 : 1	10–17	Small semi-circle particles
0.5**	2.5	0.2 : 1	18–20	Small sphere particles
0.5**	3	0.1667 : 1	10–17	Small semi-circle particles
0.5**	3	0.1667 : 1	18–20	Small sphere particles

*Sodium alginate medium viscosity solution (AMV),

**Sodium alginate low viscosity solution (ALV)

Table 5 Particle size of calcium alginate microparticles

Na alginate (%w/v)	$CaCl_2$ (%w/v)	Alginate:$CaCl_2$ (w/w)	Applied voltage (kV)	Mean diameter (nm±SD)
1*	2.5	0.4 : 1	17	1,383±155
1*	2.5	0.4 : 1	18	937±158[a]
1*	2.5	0.4 : 1	19	1,296±198
1*	3	0.33 : 1	17	1,483±192
1*	3	0.33 : 1	18	1,710±164
1*	3	0.33 : 1	19	2,330±775
0.5**	2.5	0.2 : 1	18	2,253±440
0.5**	2.5	0.2 : 1	19	4,133±135
0.5**	2.5	0.2 : 1	20	1,556±51[b]
0.5**	3	0.1667 : 1	18	1,843±312
0.5**	3	0.1667 : 1	19	3,243±914
0.5**	3	0.1667 : 1	20	2,540±653

*Sodium alginate medium viscosity solution (AMV)

**Sodium alginate low viscosity solution (ALV)

[a]Indicated F1 (1% alginate medium viscosity (AMV), 2.5% $CaCl_2$, 18 kV)

[b]Indicated F2 (0.5% alginate medium viscosity (ALV), 2.5% $CaCl_2$, 20 kV)

As shown in Table 4, with the alginate solution at lower concentrations, smaller particles were formed in all systems. The effect of the divalent cation concentration on the size and morphology of the particle revealed that $CaCl_2$ at the concentration of 3 %w/v yielded smaller sizes and more spherical shapes. The mean diameters of alginate microparticles measured by Zetasizer are shown in Table 5. The particle size was 1–2 μm depending on the type and amount of alginate. The type of sodium alginate did not affect the shape and morphology of the obtained particles. The results showed that 1 %w/v of alginate medium viscosity (AMV), 2.5 %w/v of

$CaCl_2$, electrical potential 18 kV (F1) and 0.5 %w/v of alginate low viscosity (ALV), 2.5 %w/v of $CaCl_2$, electrical potential 20 kV (F2) yielded the smallest particles of 937±158 nm and 1,556±51 nm, respectively. Decreasing the concentration (amount) of polymer resulted in the decrease in mean particle size. The results were in agreement with previous reports which revealed that particle size of poly(ε-caprolactone) nanoparticles decreased when concentrations of poly(ε-caprolactone) decreased (Benoit et al. 1999: 73–84). These might be that as polymer concentration was decreased, the surface tension force that leads to reduce size of the droplet were reduced, therefore, the size of particles was decreased. Figure 21 shows the morphology obtained from an inverted microscope at magnification of 20X of particles formulated by F1 (Figure 21A) and F2 (Figure 21B). The images appeared to be spherical, smooth and homogeneously distributed without evidence of collapsed particles. %Yield of F1 and F2 was 12% and 24% respectively.

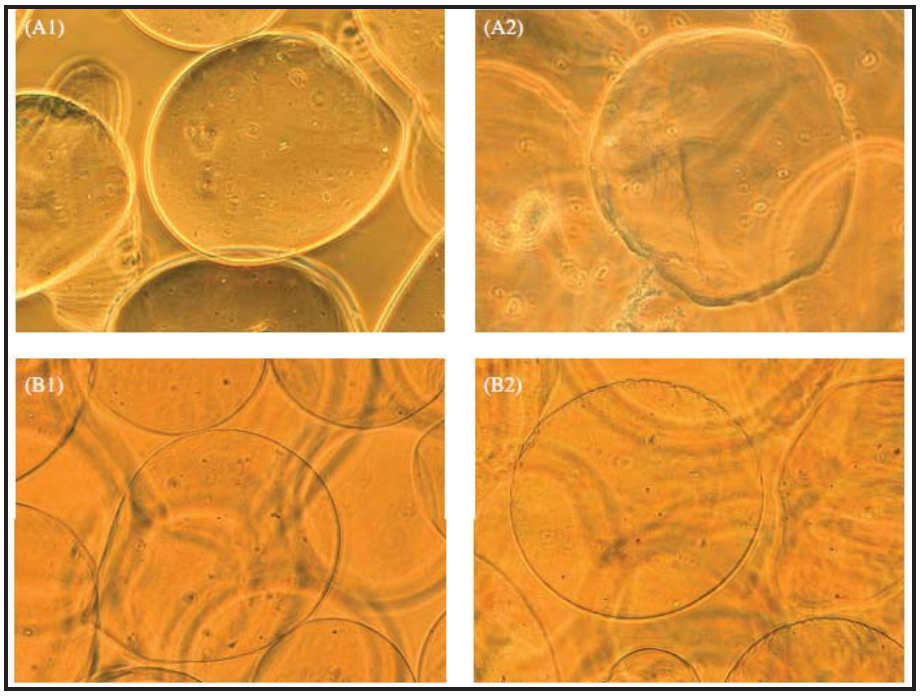

Figure 21 Microphotographs of calcium alginate microparticle obtained from an inverted-microscope at magnification of 20X; (A1) Bare-Ca-alginate microparticles of F1, (B1) Bare-alginate microparticles of F2, (A2) 40% BSA-Ca-loaded alginate micropraticles of F1, (B2) 40% BSA-loaded Ca-alginate microparticles of F2.

4.1.1.2 BSA entrapment efficiency

Two formulae (F1 and F2) were selected for BSA-loaded Ca-alginate microparticles. BSA-loaded Ca-alginate microparticles were prepared by EHDA technique. Various amounts of initial BSA (5, 10, 20, 40, 60% w/w to polymer) were incorporated into the alginate microparticles coded as F1 and F2. Aqueous solution of alginate containing BSA was dropped into $CaCl_2$ solution via applied voltage for 18–20 kV. No change in the morphology of 40% BSA-loaded microparticles was observed by an inverted-microscope (Figure 21B). Percentage yield of microparticles was found to be in the range of 6–22% (Figure 22). Due to the variation of electrospraying process that produced the microparticles in widely area, it was difficult to collect all of them. Moreover, the mucoadhesive polymer could be attached with container. For reason why, the very low amount of percentage yield of microparticles was observed. The entrapment efficiency was found to be in the range of 6–19% (F1) and 8–49% (F2), depending on the initial BSA added. F1 and F2 with initial BSA 10% w/w showed the highest entrapment efficiency of 19.13±0.01% and 49.70±0.01%, respectively, as shown in Figure 23. The results of BSA content revealed that F2 with the initial BSA of 20% w/w showed the highest amount of BSA content of 3.92±0.02 mg/g of particles followed with F1 with the initial BSA of 40% w/w as BSA content of 3.26±0.01 mg/g of particles. F2 showed higher percentage entrapment efficiency than F1. This might be due to gel formation of calcium-alginate depending on the type and the ratio of alginate/calcium ion present in the system. Degree of gelation of alginate was significant for the retention of protein inside microspheres. In this study the different viscosity of alginate and concentration were used, however concentration of calcium chloride in all formulations was constant at 2.5 %w/v, thus the ratios of alginate and calcium ions were different (F1; 0.4:1, F2; 0.2:1 w/w). The ratio of alginate and viscosity of alginate in F2 was lower than F1. The gel formation of F2 was more complete formation than F1. Therefore, percentage entrapment efficiency of F2 was higher than F1. The higher viscosity and amount of alginate in F1 might result from that amount of calcium present in the system being insufficient to complete the cross-link gel formation. The complexation of the microparticles in F1 would be high porosity, allowing more BSA to escape easily to

the gelling medium, therefore F1 showed lower percentage entrapment efficiency than F2.

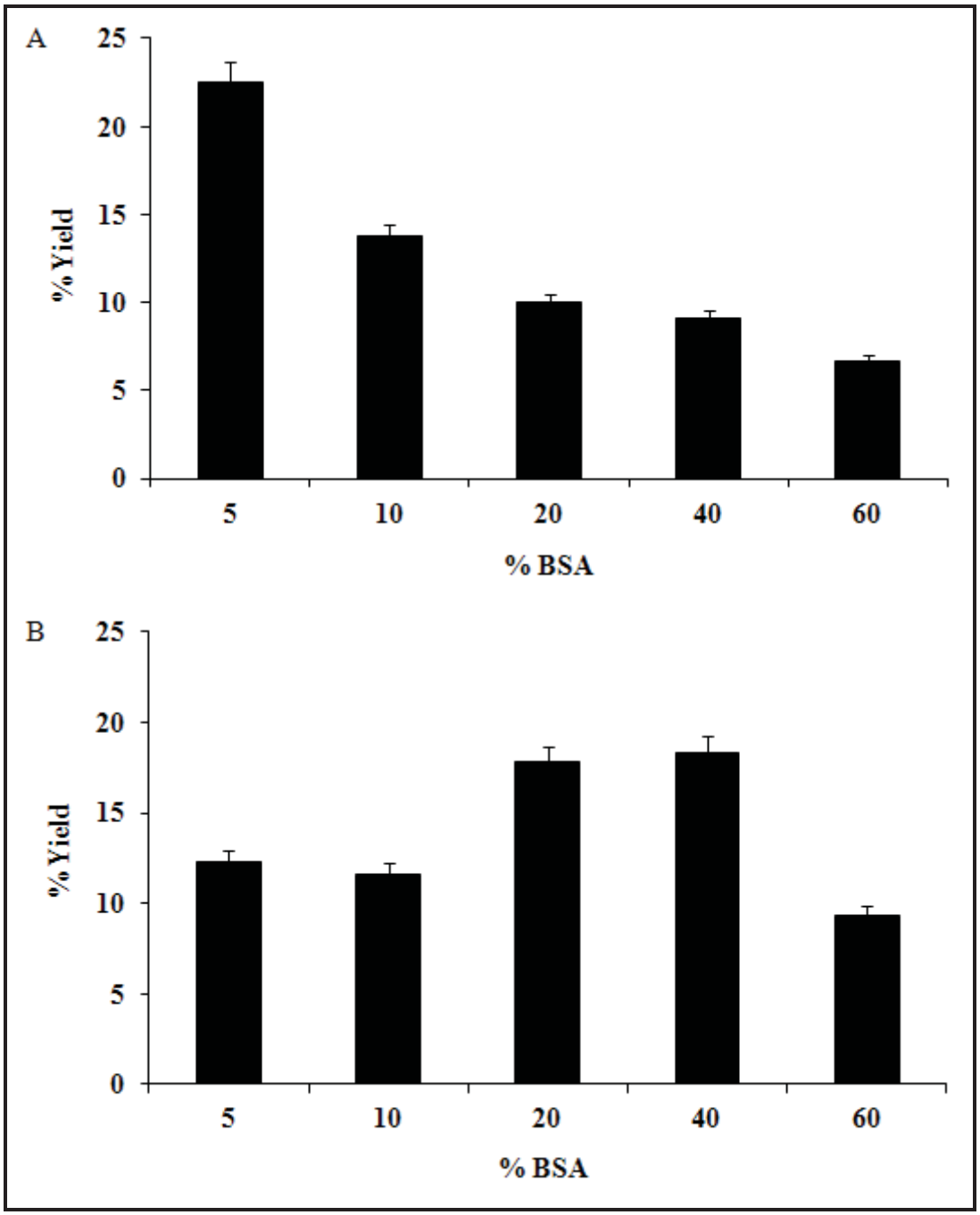

Figure 22 Effect of initial preparation concentration of BSA on percentage yield of (A) F1 and (B) F2.

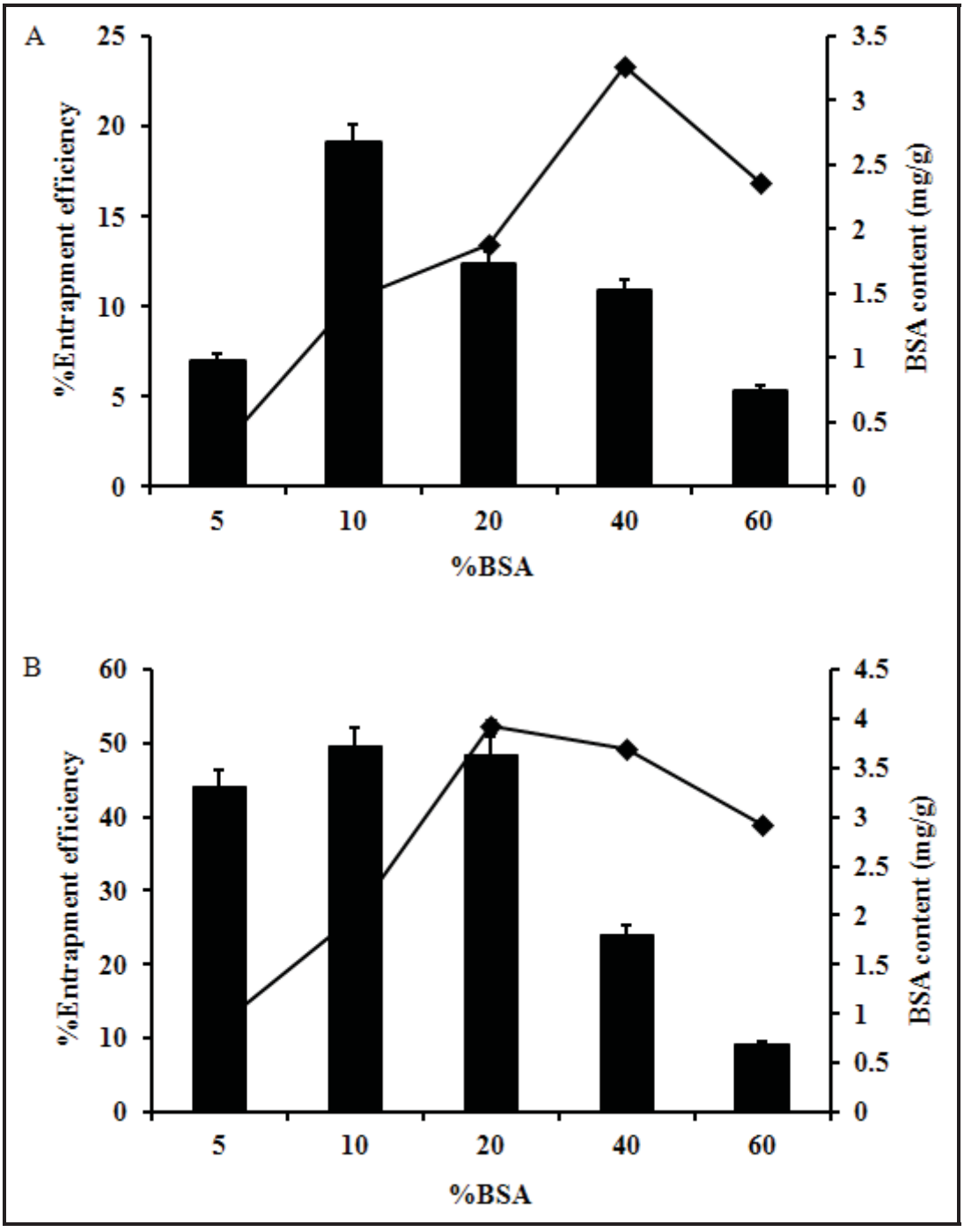

Figure 23 Effect of initial preparation concentration of BSA on (■) percentage entrapment efficiency and (♦) BSA content (mg/g) in the BSA-loaded microparticle formulation (A) F1 and (B) F2.

4.1.1.3 *In vitro* BSA release

The *in vitro* release study of BSA-loaded alginate microparticles was performed using PBS pH 7.4 and 0.1 N HCl solution (pH 1.2) as the receptor medium. The release profiles of BSA from the prepared BSA-loaded Ca-alginate microparticles F1 and F2 are shown in Figure 24.

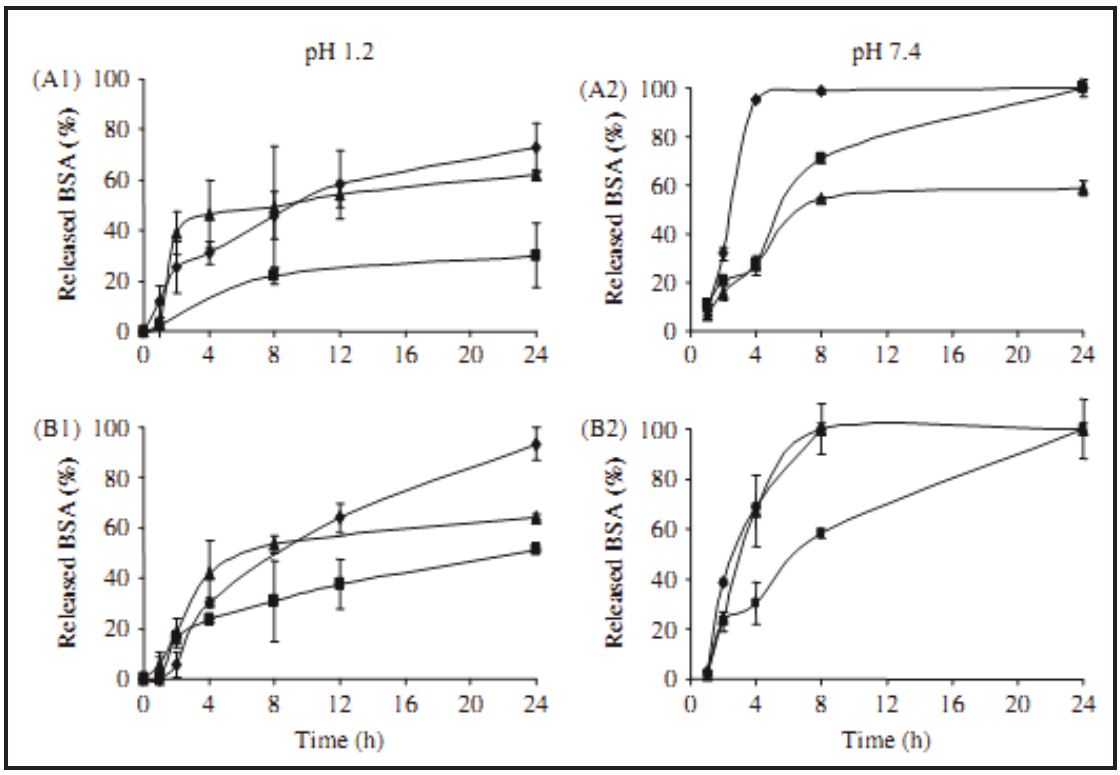

Figure 24 Release profile of BSA-loaded microparticles prepared from (A) F1 and (B) F2 in (1) HCl solution pH 1.2 medium, (2) PBS pH 7.4 medium; (♦) 5%, (■) 20% and (▲) 40% initial BSA to polymer.

Approximately 100% of BSA was released in the PBS, pH 7.4 over a period of 24 h, except for formula F1, where 40% initial of BSA was released ~ 60% at 24 h (Figure 24, A2). On the other hand, in 0.1 N HCl solution pH 1.2 receptor medium, all formulae showed slower release rate than in PBS pH 7.4 receptor medium. In comparison, the release profiles in both medium were similar. This phenomenon might be due to their macroporous structure and possible rapid dissolution at intestinal pH, which may cause low entrapment efficiency and sudden

release of core substances (George and Abraham, 2006: 1-14; Wong et al., 2002: 99-114). The slow release of BSA at final stage might be that, alginate shrinks at low pH (gastric environment), and the encapsulated drugs are not released (Chen et al., 2004: 285–300). In the gastric fluid, the hydrated sodium alginate is converted into a porous, insoluble so called alginic acid skin (Yotsuyanagi et al., 1987: 1555-1563). Release of macromolecules from microparticles in low pH solution is also significantly reduced, which could be advantageous in the development of an oral delivery system (Sugawara et al., 1994: 272–277). The release of drug from Ca-alginate microparticles took place by both diffusion through the swollen matrix and relaxation of the polymer at pH 1.2–4.5. However, the release was due to diffusion and erosion mechanisms at pH 7–7.5 (Acarturk and Takka, 1999: 291–301). The significant difference in the flux and the percentage of BSA released in the receptor compartment at 24 h can be observed in the difference initial of BSA. The higher the amount of initial BSA, the slower the releasing rate of BSA from the formulation observed.

4.1.2 Ovalbumin (OVA) loaded calcium alginate microparticles (OVA-Ca-alginate)

From preformulation of Ca-alginate microparticles, the feasibility of the EHDA technique to produce microparticles less than 10 µm of alginate cross-linked by calcium ions was investigated. The produced microparticles from this technique resulted in an appropriate size and spherical morphology with condition 1 %w/v of alginate medium viscosity (AMV), 2.5 %w/v of $CaCl_2$, electrical potential 18 kV (F1) and 0.5 %w/v of alginate low viscosity (ALV), 2.5 %w/v of $CaCl_2$, electrical potential 20 kV (F2). The %EE and *in vitro* protein release results of two formulae revealed that F2 showed higher %EE than F1 while F1 showed more sustained release than F2 (as described in section 4.1.1.2 and 4.1.1.3). The merits of two formulae results were applied by 1) using alginate low viscosity (ALV) in order to yield the high entrapment efficiency and 2) using ALV at concentration of 1%w/v to expect the sustained release of model antigen from microparticles. In case of $CaCl_2$, various concentrations of $CaCl_2$ (2.5 and 4 %w/v) were evaluated in order to adjust the morphology of microparticles in more spherical particles after loading antigen. The electrical voltage was also fixed at 20 kV. 20% BSA-loaded Ca-alginate microparticles showed in

similar entrapment efficiency and content of 40% BSA-loaded Ca-alginate microparticles. Therefore, in this study 20% OVA to polymer loaded CS-Ca-alginate microparticles was chosen to investigate the morphology by inverted microscope. Figure 25 shows microphotographs of 20% OVA loaded Ca-alginate microparticles at concentration of 2.5 and 4 %w/v of $CaCl_2$. The results revealed that using $CaCl_2$ at concentration of 4%w/v yielded the greater amount of spherical particles than concentration of 2.5%w/v. After the appropriate concentration of $CaCl_2$ was chosen, the different concentrations (10, 20 and 40% w/w to polymer) of OVA were incorporated in Ca-alginate microparticles in order to investigate the effect of entrapment efficiency, OVA content and *in vitro* release to amount of OVA entrapment in the further study.

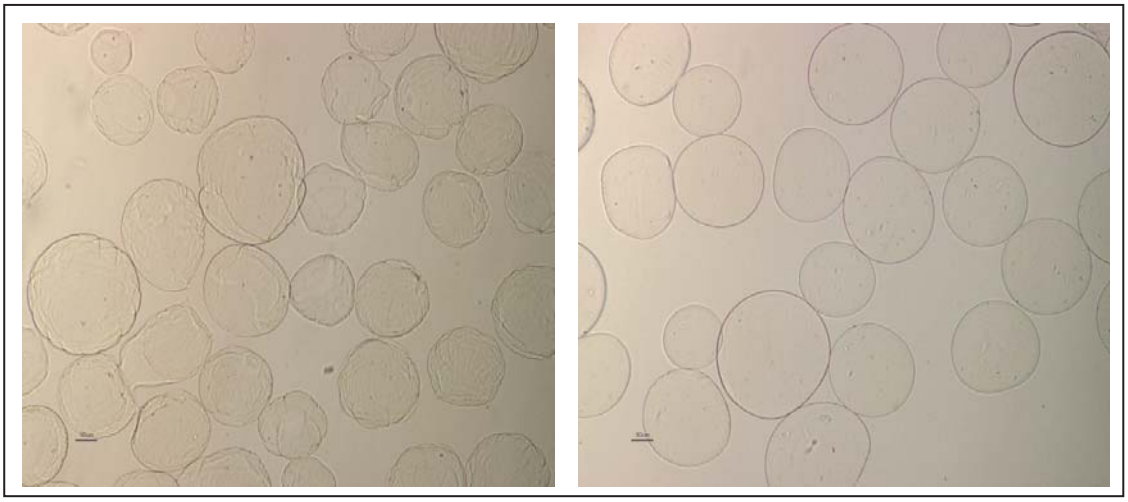

Figure 25 Microphotographs of calcium alginate microparticles obtained from an inverted-microscope at magnification of 20X; (left) 20% OVA loaded Ca-alginate microparticles at 2.5%w/v of $CaCl_2$ and (right) Ca-alginate microparticles at 4%w/v of $CaCl_2$.

In this study, OVA loaded Ca-alginate microparticles were successfully prepared by ionic gelation using EHDA technique with condition 1%w/v of ALV, 4%w/v of $CaCl_2$ and electrical voltage at 20 kV.

4.1.3 Ovalbumin (OVA) loaded calcium-yam-alginate microparticles (OVA-Ca-Yam-alginate)

The yam (Dioscorea sp.) is a local plant in many countries such as Nigeria, Jamaica, Brazil, China, and Thailand. Yam starches play a major role in the food and pharmaceutical industries over the past few decades. The yam flour has been used in bread products and snacks (Yu, Fujii and Kishihara, 1999: 5–10). In addition to being used as food, it is believed that the mixture of yam flour, rice flour, and some nutrients is beneficial to infant health. In traditional Chinese medicine, yam (*Dioscorea opposita*) tuber has been used as an important invigorant. In Thailand, the most common yam species is *Dioscorea esculenta* (Nattapulwat, Purkkao and Suwithayapan, 2009: 194-195). Yam starches show properties of tolerance to shearing and stable in acidic condition can be substituted for modified starches as a functional ingredient for low pH processed foods. The application of starch from this yam to the food industry and pharmaceutics has not been reported.

In this study, the first idea of this part, yam starch microparticles were prepared by EHDA technique using sodium trimetaphosphate (STMP) as a crosslinking agent and $CaCl_2$ as a counter ion (Table 6). The result showed an irregular form of yam particles when used yam starch, STMP and $CaCl_2$ in different concentration as shown in Table 6, F 1-3. These results indicated that no electromagnetic repulsion between electrical potential and polymer occurred because the native yam starch was non-ionic charge, whereas in EHDA technique, the negative charge of polymer must be required in order to give the same charge of applied electrical current and produce the electromagnetic repulsion (Suksamran et al. 2009). This phenomenon was effect to obstruct in spraying process. Moreover, no crosslinking process occurred when starch solution was dropped into $CaCl_2$, because the structure of starch has no guluronic acid or galactulonic acid chain that be able to form gel with divalent cation such as $CaCl_2$ (Suksamran et al. 2009, Sriamornsak et al. 2004). Therefore, in this formulation sodium alginate is the choice for choosing to incorporate with yam starch because of this polymer has a negative charge and the structure contains with guluronic group that can from gel structure with $CaCl_2$ (Gombotz and Wee, 1998: 267–285).

Table 6 Macroscopic evaluation of yam starch and Ca-Yam-alginate particles formulation

Formula	Na alginate (%w/v)	Yam (%w/v)	STMP (%w/v)	$CaCl_2$ (%w/v)	Voltage (kV)	Shape
1	-	2.5	4	4	20	Irregular
2	-	2.5	4	6	20	Irregular
3	-	2.5	6	6	20	Irregular
4	1	0	0	6	20	Small sphere particles
5	1	0.1	4	6	20	Small sphere particles
6	1	0.25	4	6	20	Small sphere particles
7	1	0.5	4	6	20	Small sphere particles
8	1	0.75	4	6	20	Small sphere particles
9	1	1	4	6	20	Small sphere particles
10	0.5	0	0	6	20	Small sphere particles
11	0.5	0.1	4	6	20	Irregular
12	0.5	0.25	4	6	20	Irregular
13	0.5	0.5	4	6	20	Irregular
14	0.25	0	4	6	20	Irregular
15	0.5	0.25	6	6	20	Small semi-circle particles
16	0.25	0.25	6	6	20	Irregular
17	0.5	0.5	6	6	20	Small semi-circle particles
18	0.5	0.25	8	6	20	Small semi-circle particles
19	0.5	0.5	8	6	20	Small semi-circle particles
20	0.5	1	8	6	20	Small semi-circle particles
21	1	0.5	4	5	20	Small sphere particles
22	1	1	4	5	20	Small sphere particles
23	1	0.5	4	4	20	Small sphere particles
24	1	1	4	4	20	Small sphere particles

The aim of this part was to investigate the conditions for microparticle formation, systems that were able to produce Ca-Yam-alginate microparticles depending on the concentration of alginate low viscosity, yam starch, STMP and $CaCl_2$ whereas the applied voltage was fixed at 20 kV (Table 6). In Table 6, the spherical particles were formed when the concentration of alginate was increased.

Whereas increasing concentration of STMP and $CaCl_2$ did not affect on the shape of particles. The minimum concentration of STMP and $CaCl_2$ resulting spherical shape of particles was found at 4 %w/v and the optimal concentration of alginate and yam starch was in ratio 1:1, in formula 24. Haider and Husain (2008: 1-6) reported that alginate-starch bead can be prepared by using alginate and starch at the concentration of 2.5 and 2.5%, respectively. These solutions were slowly extruded as droplets through a 5.0 ml syringe with attached gauge needle No. 20 into 0.2 M calcium chloride solution. The calcium alginate–starch beads were spherical in shape. From these results, the addition of hydrocolloids (alginate) to starch suspensions causes a synergistic increase in viscosity of the mixture and make this blend was more stable (Chaisawang and Suphantharika, 2005: 288–295) and the negatively charge of alginate was highly enough to form gel with $CaCl_2$. Therefore, the formula 24 was chosen to incorporate with ovalbumin (OVA) in different concentration (10, 20 and 40% w/w OVA to polymer) in the further experiment.

4.1.4 Chitosan coated calcium alginate microparticles (CS-Ca-alginate)

Coating alginate microparticles with polycationic polymers, such as chitosan, has been commonly used to control the disintegration of alginate microparticles and extend the drug release. Usually, CS-Ca-alginate microparticles are obtained by two principally different procedures: a one-step method, where a complex coacervate membrane is formed at the interface between the alginate and chitosan solutions when the alginate solution is dropped directly into a solution of calcium chloride mixed with chitosan. The other method is a two-step method, where Ca-alginate microparticles are recovered and subsequently coated with chitosan (Gaserod et al., 1998: 1815-1825). CS-Ca-alginate complex erodes slowly in phosphate buffer at pH values higher than 6.5 suppressing the initial drug release in the upper segments of the intestine occurring for uncoated microparticles and controlling the release in the colon whereas pH value is in the range of 6.5–7.0 (Tapia et al., 2004: 65-75). This behavior and degradation of chitosan by the microflora available in the colon make CS-Ca-alginate microparticles system a good candidate for effective drug delivery in the treatment of inflammatory bowel diseases (IBD) after oral administration. Moreover, this complex protects the encapsulant, has

biocompatible and biodegradable characteristics, and limits the release of encapsulated materials more effectively than either alginate or chitosan alone (Yan, Khor and Lim, 2001: 358-365). Wittaya-areekul et al. (2006: 113-118) studied the effect of the amount of chitosan able to be attached around the alginate/predinosolone core depending on the preparation method one or two step. The result showed the particle prepared by the one-step method has significantly less chitosan binding to its alginate core compared to those prepared by the two-step method. These results can be explained by the fact that when the one-step method is used, both the positively charged chitosan molecules and the positively charged calcium ions are competing with the negative charges on the surface of the alginate core. Using the two-step method firstly, the calcium ions can crosslink the alginate core surface. In the second step, the chitosan molecules can attach themselves to the left positive charge of the alginate core and the adhering chloride ions at the particle surface. By means of this second step process a thicker and well packed chitosan layer around the alginate particle is formed in comparison to the one-step method where the chitosan molecules retain their flexibility. In this study chitosan hydrochloride coated Ca-alginate microparticles were prepared by using the two-step method.

The aim of this part was to find an appropriate concentration of chitosan, which leaded to desired size ranges depending on various factors including chitosan and $CaCl_2$ concentration. Chitosan hydrochloride (CS-HCl) was chosen to coat with Ca-alginate microparticles for finding an appropriate concentration. After finding a suitable concentration of CS-HCl, chitosan derivatives were used instead of CS-HCl for coating with Ca-alginate microparticles. The mean particle size and charge of CS-Ca-alginate microparticles in various concentrations of CS-HCl are shown in Table 7.

Table 7 Particle size and zeta potential of CS-Ca-alginate microparticles with various concentrations of CS-HCl

Concentration of CS-HCl (%w/v)	Mean size (nm±SD)	Zeta potential (mV±SD)	PDI*
0	812.9±127	-7.16±0.6	0.994±0.3
0.01	954.3±135	-5.29±0.4	0.587±0.5
0.1	1121±227	12.2±0.4	0.957±0.5
0.25	1161±197	17.3±0.7	0.548±0.4

*PDI is polydispersity index

Table 7 shows the mean size and zeta potential of microparticles with various concentrations of CS-HCl. The results showed that increasing concentration of CS-HCl resulted in slightly increase in the size of microparticles, and the charge of microparticles was changed from negative charge into positive charge. As the prepared Ca-alginate microparticles was negative charge (-7.16±0.6 mV), completely coating the microparticles with CS-HCl should change to positive charge. The results revealed that the microparticles were completely coated at 0.1%w/v of CS-HCl with the net charge of 12.2±0.4 mV. As chitosan has been reported to be concentration dependent cytotoxic on Caco-2 cells, the high concentration of CS-HCl (0.25 %w/v) may damage the cells. From this reason, CS-HCl at concentration of 0.1 %w/v was chosen to coat on the surface of Ca-alginate microparticles. The morphology of Ca-alginate microparticles and CS-Ca-alginate microparticles, investigated by inverted microscopy, are shown in Figure 26 a) and b), respectively. The microparticles were apparently spherical, smooth and homogeneous distribution without evidence of collapsed particles. However, the SEM images in both microparticles were a variety and non-spherical shapes (Figure 27). These results could be explained that the microparticles were loosen in water during the freezing step before lyophilization.

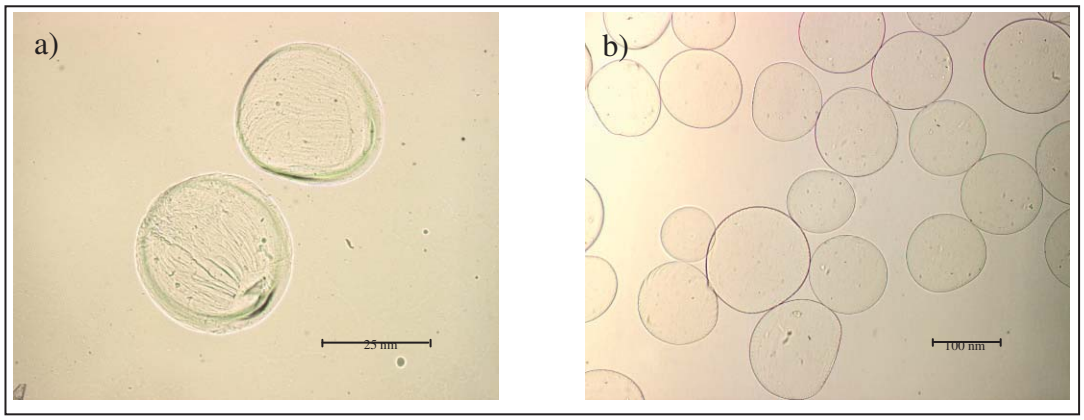

Figure 26 Microphotographs of (a) Ca-alginate microparticles and (b) CS-Ca-alginate microparticles obtained from an inverted-microscope at magnification of 40X and 20X, respectively.

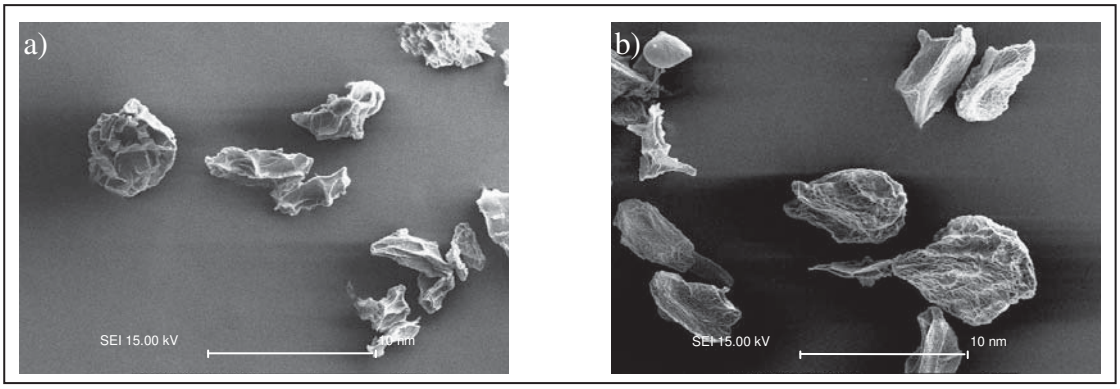

Figure 27 Scanning electron microscope (SEM) images (x500) of (a) Ca-alginate microparticles and (b) CS-Ca-alginate microparticles.

4.1.5 Water soluble chitosan derivatives coated OVA-Ca-alginate and OVA-Ca-Yam-alginate

4.1.5.1 Synthesis of chitosan derivatives

As the main drawback of chitosan (CS) is its water-insoluble property at physiological pH, CS is readily soluble in dilute acidic solutions below pH 6.0. With increasing pH, the amino groups become deprotonated and the polymer loses its charge and becomes insoluble. Versatility in the physicochemical properties of chitosan allows the formulator an excellent opportunity to engineer antigen-specific adjuvant/delivery systems. Many CS derivatives have been synthesized to enhance its

solubility, mucoadhesiveness and/or its immunostimulatory properties. Recently, the water-soluble CS derivatives, methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated *N*-(4-*N,N*-dimethylamino-cinnamyl) chitosan (TM-CM-CS) and methylated *N*-(4-pyridinylmethyl) chitosan (TM-Py-CS) were successfully synthesized and showed mucoadhesive properties and *in vitro* absorption enhancing properties (Sajomsang et al., 2009: 945–952; Kowapradit et al., 2008: 1143–1152; 2010: 301–312). Based on these results, the aim of this part was to further investigate the feasibility of applying these water-soluble CS derivatives as an adjuvant for inducing immune responses to ovalbumin (OVA), the model antigen, via the oral route. The percentage of degree of quaternization (DQ) and extent of *N*-substitution (ES) of these water-soluble chitosan derivatives used in this study were considered from previous report (Kowapradit et al., 2011: 430–437) as shown in Table 8. The chemical structures of water-soluble chitosan derivatives are shown in Figure 28.

Table 8 Methylation of chitosan and *N*-aryl chitosan derivatives

Samples	ES (%)	DQ_T(%)		$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Total *O*-CH_3 (%)	FW
		DQ_{Ar}(%)	DQ_{CS}(%)				
$TM_{65}CS$ (TM)	-	-	65	23	Trace	35	221
$TM_{65}CM_{50}CS$ (CM)	50	50	15	24	Trace	15	255
$TM_{56}Bz_{42}CS$ (Bz)	42	42	14	2	17	5	233
$TM_{53}Py_{40}CS$ (Py)	40	40	13	2	7	5	232

ES is the extent of *N*-substitution; DQ_{Ar} is the degree of quaternization at aromatic substituents; DQ_{CS} is the degree of quaternization at the primary amino groups of chitosan; DQ_T is the total degree of quaternization (DQ_{Ar} + DQ_{CS}); $N(CH_3)_2$ is *N,N*-dimethylation; $NHCH_3$ is *N*-methylation; Total *O*-CH_3 is the total degree of *O*-methylation of 3-*O* and 6-*O* at 3-hydroxyl and 6-hydroxyl positions of GlcN of chitosan, respectively; FW is the formular weight.

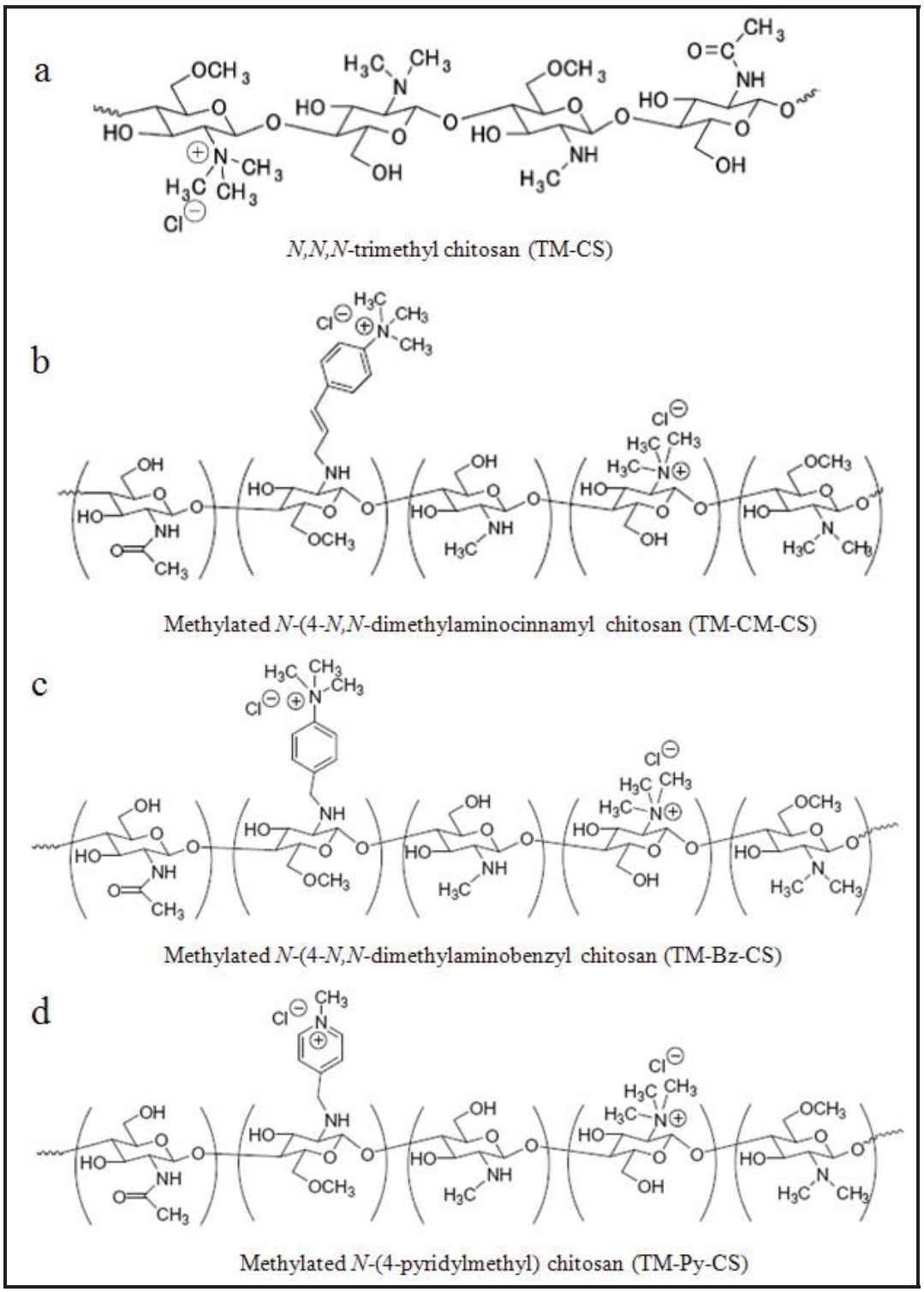

Figure 28 Chemical structures of chitosan derivatives: (a) *N,N,N*-trimethyl chitosan (TM), (b) methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan (CM), (c) methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (Bz), (d) methylated *N*-(4-pyridylmethyl) chitosan (Py).

4.1.5.2 Cytotoxicity of chitosan derivatives

Cytotoxicity of water-soluble chitosan derivatives was investigated by MTT assay. The modified chitosan was determined as cationic polymers that have been known to be cytotoxic materials. The results showed that all chitosan derivatives tested showed concentration-dependent cytotoxicity in Caco-2 incubated for 24 h at pH 7.4 (Table 9). Cytotoxicity of chitosan derivatives were in the following order: $TM_{53}Py_{40}CS > TM_{56}Bz_{42}CS > TM_{65}CM_{50}CS \sim TM_{65}CS$. These results suggested that addition of trimethyl groups on the cinnamyl moiety showed high cytotoxicity as well as the methylated CS, whereas addition of trimethyl and methyl groups on the benzyl and pyridyl moieties in the polymer structure could reduce the cytotoxicity in Caco-2 cells as shown by the increase in IC_{50} value at 24 h.

Table 9 Cytotoxicity of methylated *N*-aryl chitosan derivatives incubated with Caco-2 cells for 24 h (n=8)

Chitosan derivatives	pH	IC_{50} (mg/ml) at 24 h
$TM_{65}CS$ (TM)	7.4	0.03±0.01
$TM_{65}CM_{50}CS$ (CM)	7.4	0.03±0.01
$TM_{56}Bz_{42}CS$ (Bz)	7.4	0.34±0.01
$TM_{53}Py_{40}CS$ (Py)	7.4	0.69±0.08

4.1.5.3 Adjuvant activity of chitosan derivatives

It has been shown that CS solutions could enhance the immunoadjuvant properties of cytokines when co-administered subcutaneously. CS could also enhance the antigen-presenting capability of dendritic cells and induce greater allogeneic T-cell proliferation. Moreover, CS and its derivatives, TMC, exhibit immunoadjuvants and antigen delivery systems for mucosal vaccinations (Boonyo et al., 2007: 168–175). The adjuvant activity of these modified CS was also explored by immunizing the mice with the various formulations as shown in Table 10.

Table 10 Immunization formulations in mice groups A–D, P and N

Mice groups	Formulations	Administration
P	100 μg of OVA in PBS, pH 7.4 with $Al(OH)_3$	S.C.
N	500 μg of OVA in PBS, pH 7.4	P.O.
A	500 μg of OVA in 0.1% w/v $TM_{65}CS$ solution	P.O.
B	500 μg of OVA in 0.1% w/v $TM_{65}CM_{50}CS$ solution	P.O.
C	500 μg of OVA in 0.1% w/v $TM_{56}Bz_{42}CS$ solution	P.O.
D	500 μg of OVA in 0.1% w/v $TM_{53}Py_{40}CS$ solution	P.O.

4.1.5.3.1 OVA-specific immunoglobulin G (IgG) antibody

Adjuvant activity of the methylated *N*-aryl chitosan derivatives was determined by measurement OVA-specific serum immunoglobulin G (IgG) antibody. IgG titers at day 0 were very low baseline but the IgG titers following the second booster were significantly increased (Figure 29). The results showed that, on day 21, significant difference of IgG levels was observed in group P and group B compared with group N. However, IgG levels in those CS derivative solutions were not significantly difference compared with group N. Comparing the results of CS derivatives, IgG levels of mice those received OVA in $TM_{65}CM_{50}CS$ were significantly higher than those received OVA in $TM_{65}CS$, $TM_{56}Bz_{42}CS$ and $TM_{53}Py_{40}CS$. These results indicated that at the equal concentration of CS derivatives (0.1%w/v), OVA in $TM_{65}CM_{50}CS$ could elicit higher IgG responses than OVA in the other CS derivatives. Most significant sera collected from the $TM_{65}CM_{50}CS$ immunized mice showed greater IgG responses than those collected sera from $TM_{65}CS$ (16 times), $TM_{56}Bz_{42}CS$ (10 times) and $TM_{53}Py_{40}CS$ (7 times).

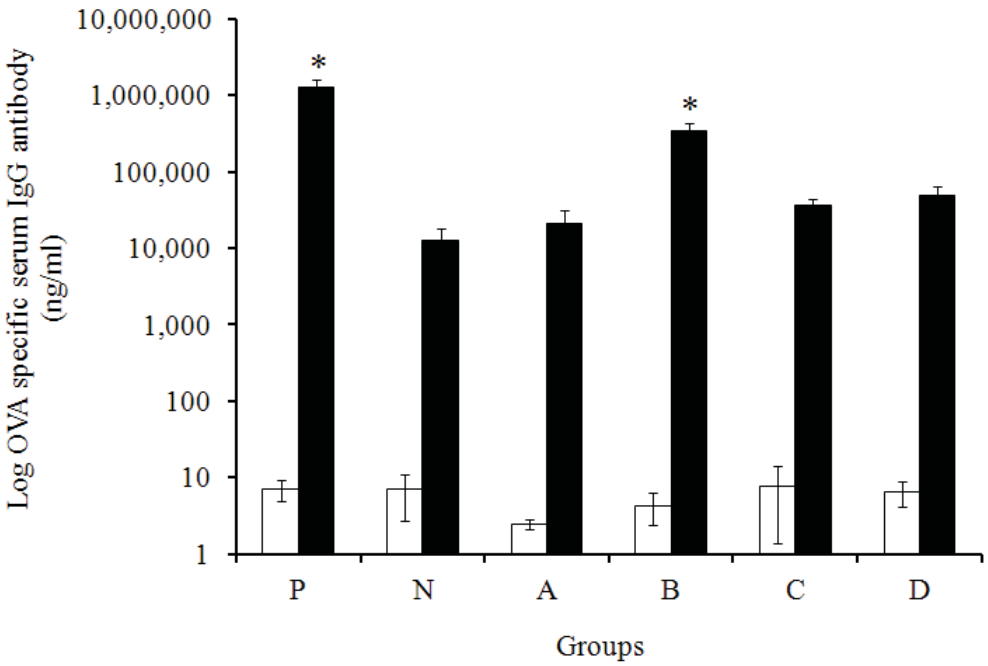

Figure 29 Serum IgG antibody titers at day 0 (white bars) and at day 21 (black bars) obtained from mice following oral immunization with OVA in PBS (group N), $TM_{65}CS$ (group A), $TM_{65}CM_{50}CS$ (group B), $TM_{56}Bz_{42}CS$ (group C), $TM_{53}Py_{40}CS$ (group D), and in alum (group P) administered subcutaneously (positive control) (n=6). Significant differences with negative control groups were designated as ∗ ($p<0.05$).

The results indicated that mice those received OVA with chitosan derivatives could increase immune response when compared with those received OVA alone. These results it can be explained by TEER properties of chitosan. It is well known that chitosan solutions cause a significant and dose-dependent decrease of TEER of the Caco-2 cell monolayers by acting on negatively charged sites at the cell surfaces and tight junctions, and it has been shown that chitosan is able to induce changes in F-actin distribution (Artursson, 1992: 1358-1361). The interaction of CS with the cell membrane results in a structural reorganization of tight junction-associated proteins, followed by enhanced transport through the paracellular pathway. Therefore, binding of CS to the Caco-2 cells precedes absorption enhancement, and this increase in absorption is mediated by the positive charges on the polymer (Schipper, 1997: 923–929). The result of adjuvant activity of chitosan derivatives was

correlated with the result of TEER in previous study which showed that CS containing aromatic *N*-dimethylaminobenzyl and *N*-dimethylaminocinnamyl groups, decreased of TEER values and increased FD-4 transport. (Kowapradit, 2010: 301–312). However, IgG titer of $TM_{56}Bz_{42}CS$ group was not significantly different from the control group as well as $TM_{53}Py_{40}CS$ and $TM_{65}CS$ groups. This finding could be explained that $TM_{65}CM_{50}CS$ appeared to be more toxic than those modified CS. Hence, at the same concentration of polymers, $TM_{65}CM_{50}CS$ had a great effect on tight junction permeability. Due to the different chain length between CS backbone and quaternary ammonium moieties of the TM-Bz-CS and TM-CM-CS, it was postulated that TM-CM-CS would tightly bind to negatively charged sites more than those of TM-Bz-CS at the cell surfaces and tight junctions followed by enhancing the transport through the paracellular pathway. From *in vitro* studies, it could be explained why $TM_{65}CM_{50}CS$ had a better effect on adjuvant activity *in vivo* study than $TM_{56}Bz_{42}CS$. In case of $TM_{53}Py_{40}CS$ which did not affect on tight junction permeability and was not significantly different in immune response after oral administration, it could be possible that the steric hindrance of the *N*-pyridylmethyl group shielded the positive charges of the quaternary ammonium group on the GlcN of CS, and resulted in hindering the binding of the polymers to negatively charged sites at the cell surfaces and tight junctions. Moreover, the positive charge in the pyridine ring could be delocalized by the resonance effect, while the positive charges in methylated chitosan derivatives were fixed. The adjuvant activity of $TM_{65}CS$ was the lowest. This result was in agreement with the observations of a previous study which showed that TMC with 60% of DQ showed a low immune response and no significant difference in comparison to control and lower DQ of TMC groups (Boonyo et al., 2007: 168-175). In addition, although cytotoxicity of $TM_{65}CS$ was similar to $TM_{65}CM_{50}CS$, its absorption enhancing property and adjuvant activity were lower. The mechanism of these CS derivatives for enhancement of immunogenicity via oral route may be caused from the interaction between CS and the cell membrane, resulting in a structural reorganization of tight junction-associated proteins, followed by enhancing the transport through the paracellular pathway and increasing the antigen absorption. The ability of soluble CS to adjuvant activity is also related to its mucoadhesive property, which increases interpenetration of the mucoadhesive

molecules into the mucus glycoproteins (Schipper et al., 1997: 923–929). In previous study, these CS derivatives (TM-CS and TM-CM-CS) showed the mucoadhesive property, depending on the DQ and polymer structure. When the DQ was higher than 65%, the $TM_{65}CM_{50}CS$ had a similar mucoadhesive property to $TM_{65}CS$ (Sajomsang, Ruktanonchai and Nuchuchua, 2009: 945–952). Moreover, $TM_{65}CM_{50}CS$ could protect the degradation of bovine serum albumin (BSA) when it was co-administered with BSA, and incubated with simulated intestinal fluid containing 1% w/v pancreatin porcine pancreas. These studies indicated that the adjuvant effect of these CS derivatives might be from the combination of the protection of antigen degradation from the gastrointestinal tract fluid (Kowapradit et al., 2012: 465-468), the induction of mucoadhesive effects (Sajomsang, Ruktanonchai and Nuchuchua, 2009: 945–952), and the enhancement of paracellular transport. In general, there are two distinctive pathways to allow the transport of antigen into the lymphoid tissue, depending on the nature of antigen. Soluble antigen is ables to penetrate the intestinal epithelium into the lamina propria (LP), and interact with the antigen presenting cells (APCs) such as macrophages and dendritic cells. The APCs migrate to the lymph node where the antigen is presented to the T cells as a start of the activation of the IgG immune response cascade. In contrast, antigen in particulate form is largely taken up by M-cells for transportation to gut-associated lymphoid tissue (GALT), and is subsequently transferred to underlying APCs for the initiation of antigen-specific mucosal sIgA and IgG responses (Boontha et al., 2011: 43-47; De Magistris, 2006: 52-67). Moreover, Seferian et al inoculated BALB/c mice with chitosan plus β- human chorionic gonadotropin, and found that the mixed immune response to IgG1, IgG2a and IgG2b antibodies could be observed in the groups with chitosan emulsion as adjuvant by intraperitoneal injection (Bivas-Benita et al., 2003: 17-27). Bivas-Benita et al immunized mice with oral Toxoplasma gondii GRA1 protein and DNA vaccine-loaded chitosan particles, and specialized anti-GRA1 IgG1 and IgG2a were successfully induced, indicating that immune respons to Th1 and Th2 could be enhaced (Xie et al., 2007: 1547-1553). Xie et al revealed that *H. pylori* with chitosan solution as an adjuvant can protect *H. pylori* infection and induce both Th1 and Th2 type immune response by oral. Therefore, the type of immune response of these CS derivatives requires further investigation.

4.1.5.3.2 OVA-specific immunoglobulin A (IgA) antibody

Figure 30 shows IgA antibody titers that obtained from mice those were recieved oral immunization with chitosan derivatives solution. The results showed that IgA levels obtained from the mice those received OVA in $TM_{65}CM_{50}CS$ solution (Group B) were higher than those received OVA in other derivatives. These results indicated that OVA in $TM_{65}CM_{50}CS$ solution could initiate higher IgA response than OVA in saline solution, OVA in $TM_{65}CS$ solution, OVA in $TM_{56}Bz_{42}CS$ solution and OVA in $TM_{53}Py_{40}CS$ solution. When mice were immunized s.c. with OVA in alum. Very little IgA responses were observed, and it was correlated with IgG level. It could be concluded that most of OVA/chitosan derivatives preparations enhanced mucosal immune responses in comparison to the positive and negative control. As the antigens incorporated in chitosan derivatives solutions are predominantly taken up by direct transport across the mucosal tissue and not via an M-cell mediated uptake (Davis, 2001: 21–42), the ability of the polymers to reversibly open the tight junctions of the mucosal tissue may become an important aspect of the efficacy of antigen uptake (Boonyo et al., .2007: 168-175). For this reason, chitosan derivatives can induce the immune response.

The aim of this study was to characterize the novel chitosan derivatives including $TM_{65}CS$, $TM_{65}CM_{50}CS$, $TM_{56}Bz_{42}CS$ and $TM_{53}Py_{40}CS$. The cytotoxicity and immunogenicity of these chitosan derivatives were also investigated. The results showed that OVA in $TM_{65}CM_{50}CS$ solution could induce the highest IgG and IgA levels in mice. Therefore, $TM_{65}CM_{50}CS$ could be used for coating the microparticles. Then characterization, entrapment efficiency, *in vitro* release and *in vivo* immune response study of coated and uncoated microparticles were investigated.

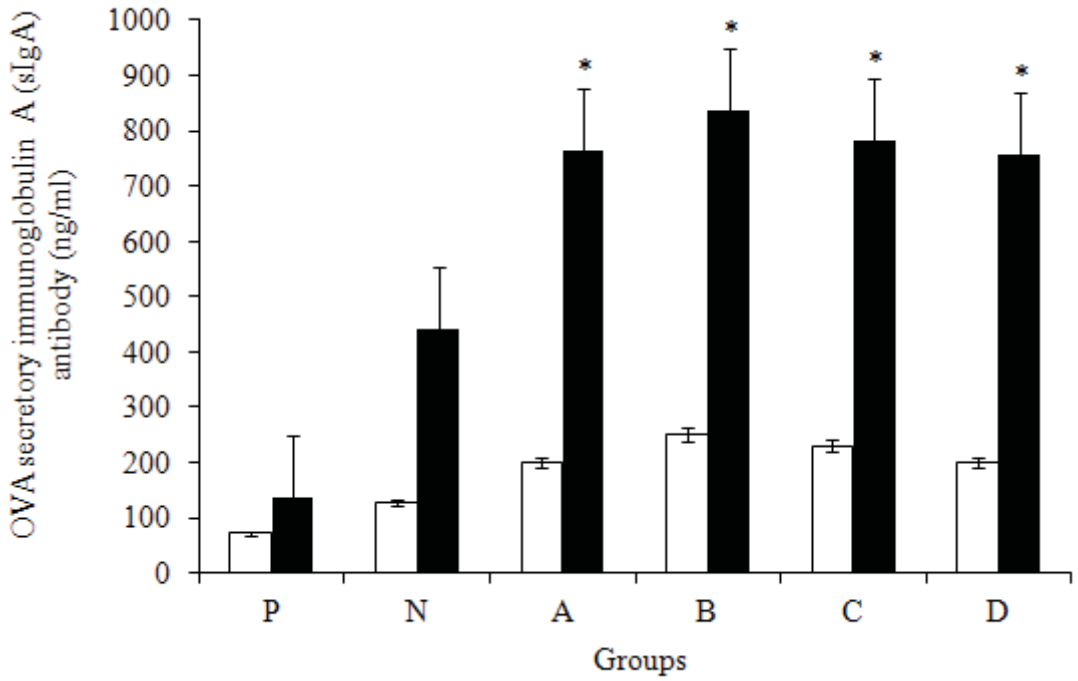

Figure 30 IgA antibody titers at day 0 (white bars) and at day 21 (black bars) obtained from mice following oral immunization with OVA in PBS (group N), $TM_{65}CS$ (group A), $TM_{65}CM_{50}CS$ (group B), $TM_{56}Bz_{42}CS$ (group C), $TM_{53}Py_{40}CS$ (group D), and in alum (group P) administered subcutaneously (positive control) (n=6). Significant differences with negative control groups were designated as ∗ ($p<0.05$).

4.2 Characterization of OVA loaded microparticles

OVA loaded microparticles including Ca-alginate microparticles, Ca-Yam-alginate microparticles, CM-Ca-alginate microparticles and CM-Ca-Yam-alginate microparticles were prepared in the same manner as bare microparticles with initial concentration 20% w/w to polymer of OVA (as described in section 4.1.2). The preparation of chitosan derivatives, 0.1 %w/v of $TM_{65}CM_{50}CS$, coated microparticles was followed the method of CS-HCl coated Ca-alginate microparticles. Their physicochemical properties were investigated as described below.

4.2.1 Particle size and zeta potential

Microparticles were prepared in the same procedure according to preparation of microparticles (as described in section 4.1). The mean particle size and zeta potential of microparticles are shown in Table 11. The results showed that Ca-alginate microparticles loaded/unloaded OVA yielded the smallest particles of 812.9±117 nm for loaded OVA and 797.9±108 nm for unloaded OVA with a negative charge of 7.16±0.6 and 13.6±0.5, respectively. After incorporation with yam starch (1%w/v), the size of particles was increased to 1,797±128 nm and 1,690±138 nm with a negative charge of 12.00±0.5 and 15.09±0.5 for loaded and unloaded OVA, respectively. Before OVA loading, Ca-alginate and Ca-Yam-alginate microparticles showed a slightly negative zeta potential. However, after the incorporation of OVA, more negative zeta potential was obtained (Table 11).

Table 11 Particle size and zeta potential of microparticles with and without 20 % w/w to polymer of OVA

Microparticles	Mean size (nm±SD)	Zeta potential (mV±SD)	PDI*
Ca-alginate	812.9±117	-7.16±0.6	0.994 ± 0.3
OVA-Ca-alginate	797.9±108	-13.60±0.5	0.742± 0.4
CM-Ca-alginate	1387±208	12.91±0.4	0.882± 0.6
OVA-CM-Ca-alginate	1491±109	10.93±0.5	0.444± 0.3
Yam-Ca-alginate	1797±128	-12.00±0.5	0.495± 0.5
OVA-Yam-Ca-alginate	1690±138	-15.90±0.5	0.841± 0.5
CM-Ca-Yam-alginate	2590±222	12.61±0.5	0.630± 0.4
OVA-CM-Ca-Yam-alginate	2960±224	6.03±0.7	0.601± 0.4

This is probably due to the negative charge of OVA. This result was corresponded with previous report of Beatriz et al (2008: 98–108). They reported that

CpG oligonucleotide Th1-biased adjuvant activity can be improved when closely associated with a variety of antigens in PLGA microparticles. They found that the zeta potential was moved towards slightly negative when CpG motifs were encapsulated 3.1±0.8 to -5.1±0.3 mV. In case of coated particles, CM-Ca-alginate and CM-Ca-Yam-alginate microparticles, loaded and unloaded OVA into microparticles yielded in spherical shape with mean diameter of 1,387±208, 1,491±109 and 2,960±224, 2,590±222 nm, respectively. The zeta-potential of microparticles was positive charge of 12.91±0.4 and 10.93±0.5 mV. Due to the contribution of the $TM_{65}CM_{50}CS$, a positively charge of coated microparticles was observed.

4.2.2 Morphology

4.2.2.1 Ca-alginate microparticles

The SEM images of microparticles are shown in Figure 31. All particles were smooth-surfaced, however after freeze drying the particles appeared in wither shape. These results could be explained that the water inside microparticles were loosen during the freezing step before lyophilization. The freezing step is significantly important during lyophilization because it is the main desiccation step. Moreover, the freezing procedure directly impacts ice crystal formation and thus product morphology (Kasper and Wolfgang 2011,: 248–263).

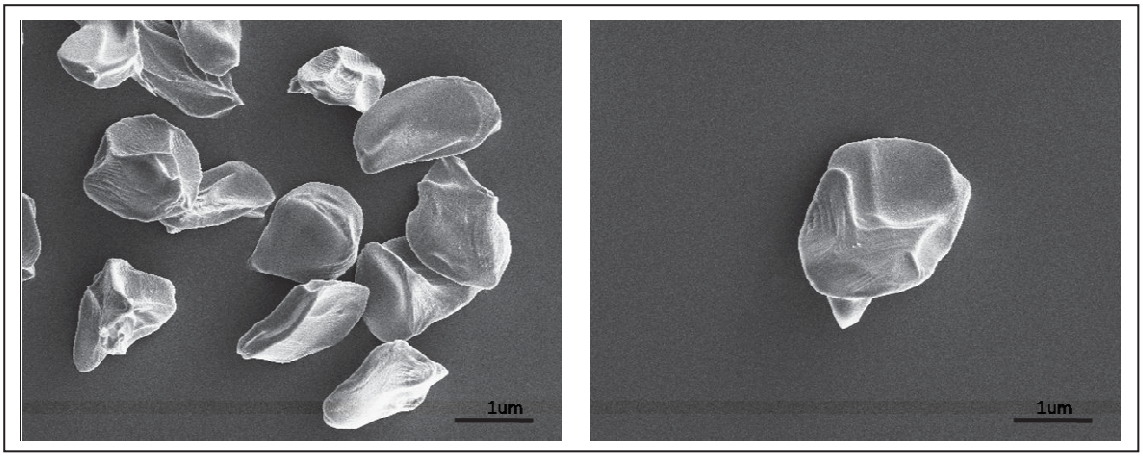

Figure 31 Scanning electron microscope (SEM) images of calcium alginate microparticles; left x100 and right x1500.

4.2.2.2 Ca-Yam-alginate microparticles

Figure 32 shows the morphology of Ca-Yam-alginate microparticles (a) and OVA loaded Ca-Yam-alginate microparticles (b). The images of bare Ca-Yam-alginate microparticles appeared to be spherical, smooth and homogeneously distributed without evidence of collapsed particles however after loaded with OVA the particles had a little bit collapsed but still showed spherical sphere. The SEM images of microparticles are shown in Figure 33. All the particles were smooth-surfaced spheres but also flattened, disk-shaped particles. The absence of ideal spherical morphology can be probably attributed to the drying process that causes certain invaginations in the particles (Fig. 33a). The particles tended to agglomerate (Fig. 33b), probably due to the specific localization of the polymers and existence of attractive electrostatic forces. The size of Ca-Yam-alginate microparticles was larger than Ca-alginate microparticles as yam starch was added in their formulae. However, the size of Ca-Yam alginate microparticles was still in acceptable range size (1-5 µm) for uptake and translocation of vaccine via the oral route (Rice-Ficht et al. 2010: 106 – 112).

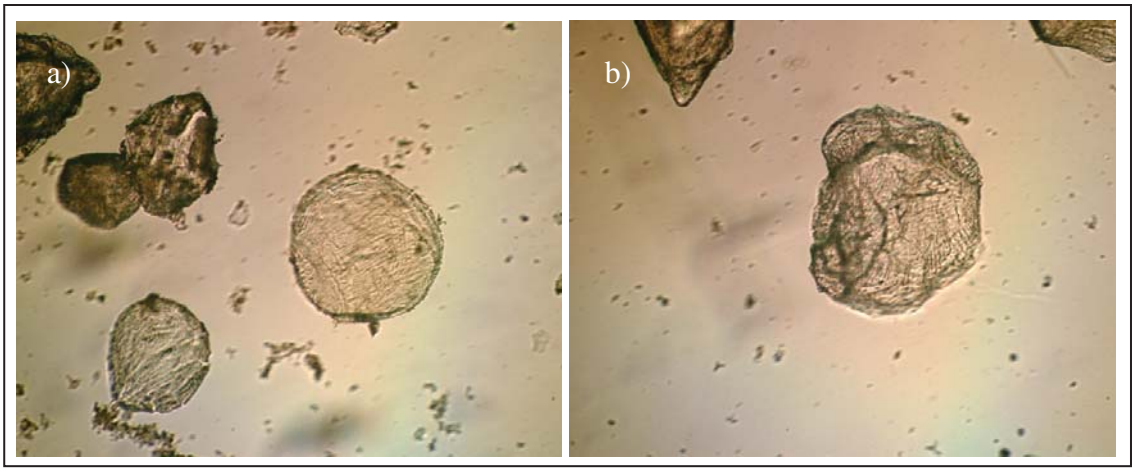

Figure 32 The morphology of (a) Ca-Yam-alginate microparticles and (b) OVA loaded Ca-Yam-alginate microparticles by inverted microscope (20X).

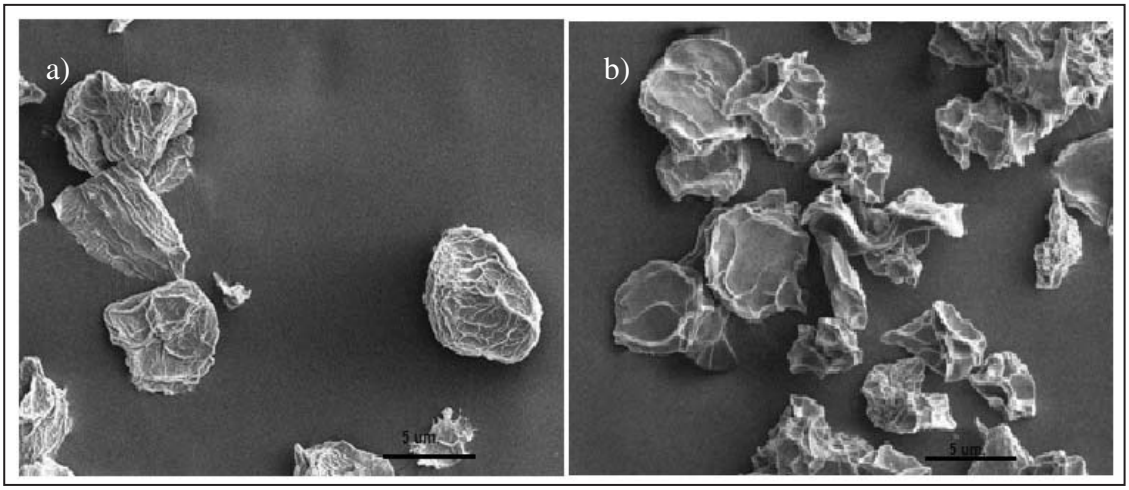

Figure 33 Scanning electron microscope (SEM) images (x500) of (a) Ca-Yam-alginate microparticles and (b) OVA-Ca-Yam-alginate microparticles.

4.2.2.3 CM-Ca-alginate microparticles

Figure 34 and 35 show the morphology of (a) CM-Ca-alginate microparticles and (b) OVA loaded CM-Ca-alginate microparticles obtained from an inverted-microscope and SEM, respectively. A spherical morphology of microparticles was observed.

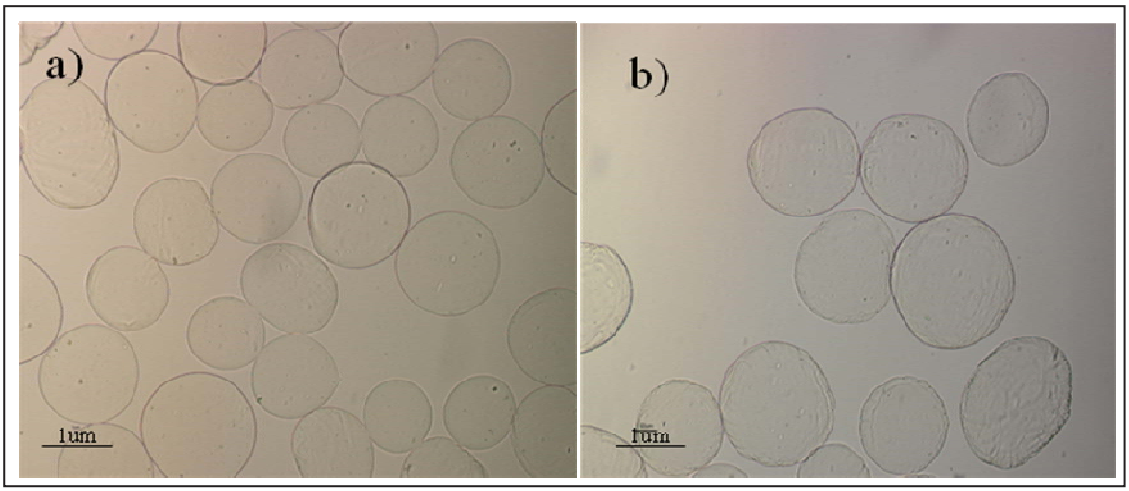

Figure 34 Microphotographs of (a) CM-Ca-alginate microparticles and (b) OVA loaded CM-Ca-alginate microparticles obtained from an inverted-microscope at magnification of 20X.

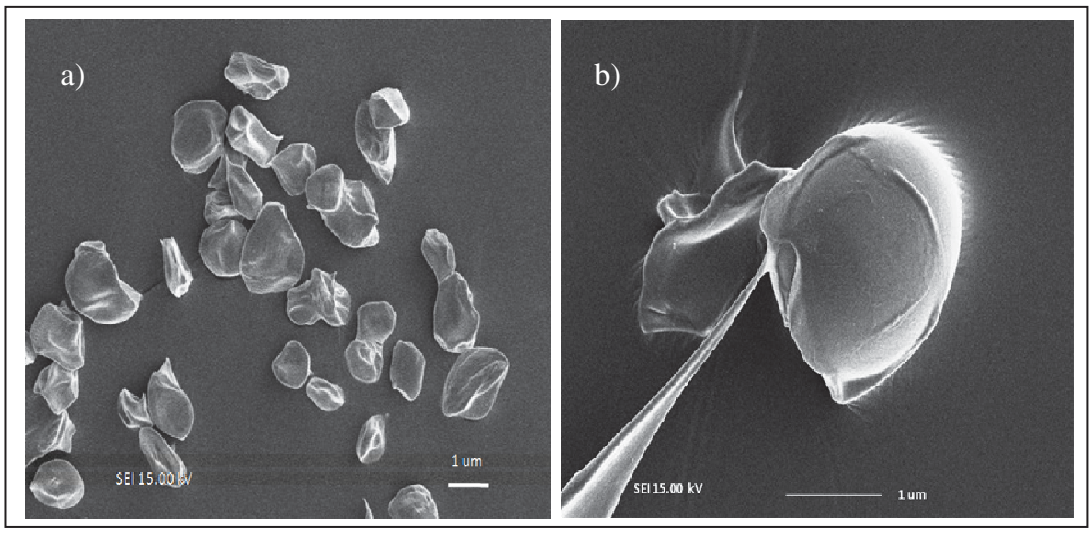

Figure 35 Scanning electron microscope (SEM) images of (a) CM-Ca-alginate microparticles (x500) and (b) OVA-CM-Ca-alginate microparticles (x1000).

4.2.2.4 CM-Ca-Yam-alginate microparticles

Figure 36 and 37 show the morphology of (a) CM-Ca-Yam-alginate microparticles and (b) 20% OVA loaded CM-Ca-Yam-alginate microparticles obtained from an inverted-microscope and SEM, respectively. Freeze dried CM-Ca-Yam-alginate microparticles showed in sponge-like structure, and remained almost spherical shape. However, the shape of microparticles was recovered into spherical shape when resuspended in PBS solution.

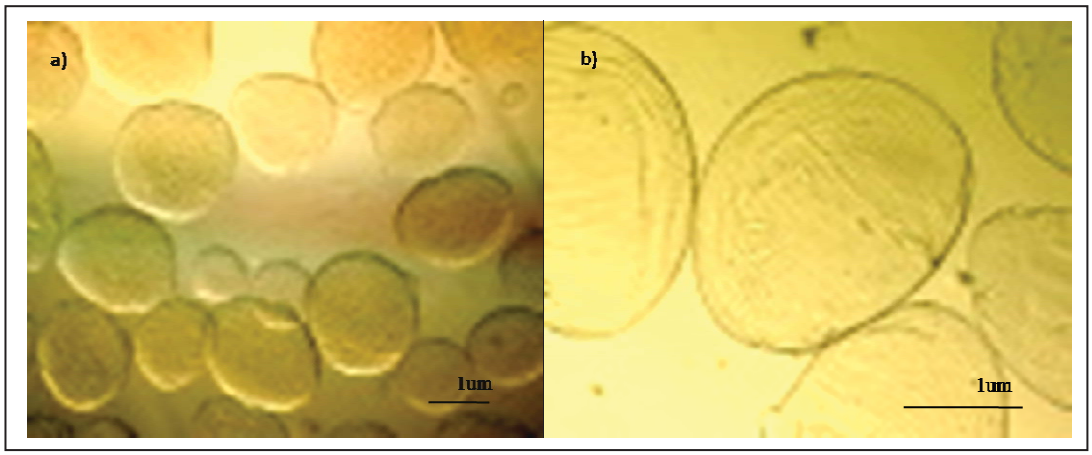

Figure 36 The morphology of (a) CM-Ca-Yam-alginate microparticles and (b) 20% OVA loaded CM-Ca-Yam-alginate microparticles by inverted microscope at magnification of 20X and 40X, respectively.

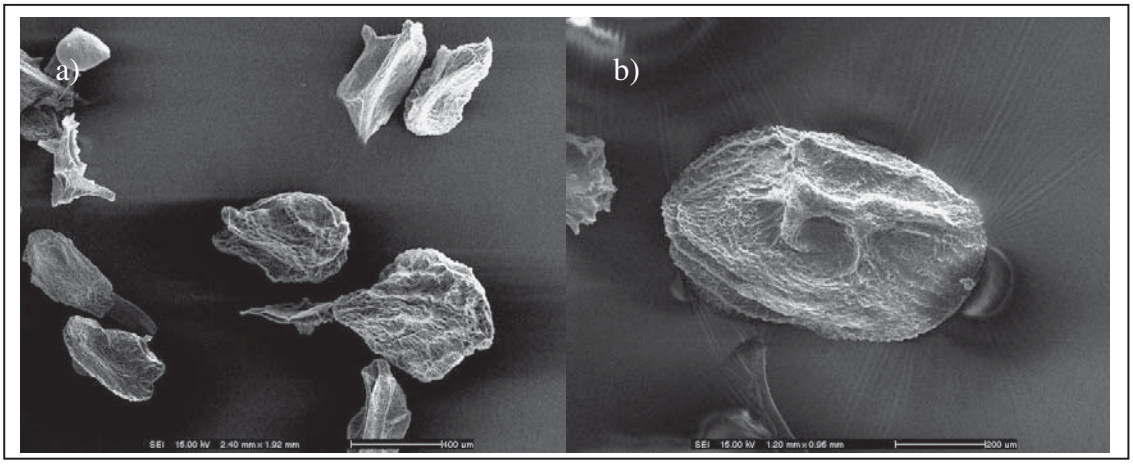

Figure 37 Scanning electron microscope (SEM) images of (a) CM-Ca-Yam-alginate microparticles (x500) and (b) OVA loaded CM-Ca-Yam-alginate microparticles (x1000).

4.2.3 Chemical characterization

4.2.3.1 Ca-alginate microparticles

According to the characteristic FT-IR spectra of the microparticles, an attempt was made to elucidate the eventual existence and type of interactions between the polymers and the drug. Sodium alginate as a carboxylate salt showed a strong asymmetric stretch at 1605 cm^{-1}. The frequency of carbonyl absorption is lower compared to the value found for the parent carboxylic acid due to a resonance phenomenon. The carboxyl and carboxylate groups are present at wave number of about 1000–1400 cm^{-1} (Borges et al., 2005: 155–166). The characteristic alginate spectra have been previously described (Wang and He, 2002: 117–126; Soares et al., 2004: 57–64) and they correspond to CH (2950 cm^{-1}), COO (1613 cm^{-1}), CH (1415 cm^{-1}) and C-O-C (1033 cm^{-1}). The FT-IR spectra of Ca-alginate microparticles showed that the absorption band around 2950, 1620, 1438 and 1040 cm^{-1} corresponds to the stretching of -CH, COO-, -CH and C-O-C, respectively, while the band at 3440 cm^{-1} belongs to the stretching vibrations of the hydroxyl groups bonded via hydrogen bonds (Figure 38).

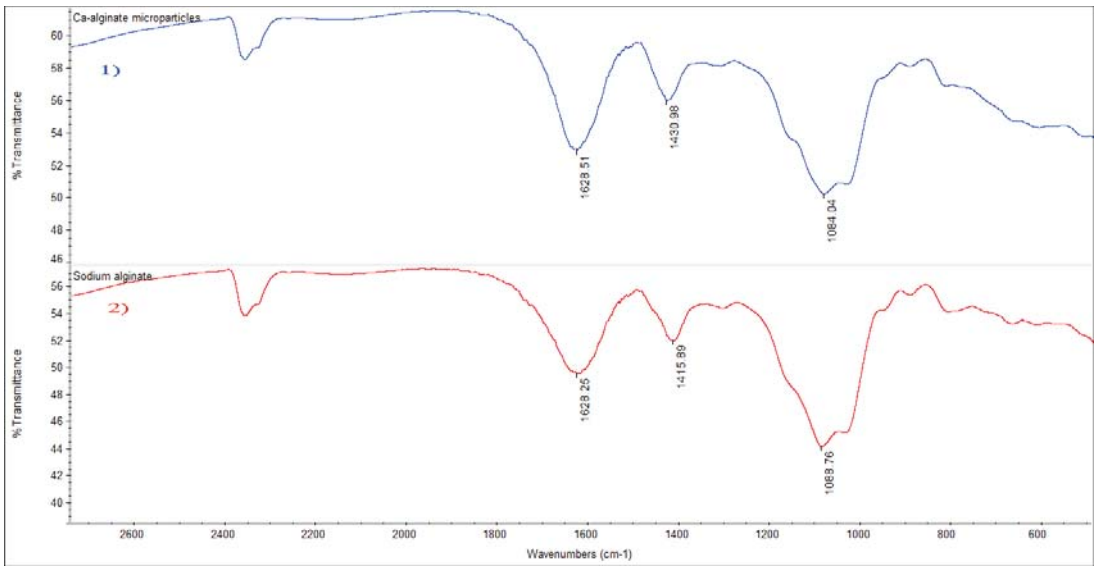

Figure 38 The FT-IR (KBr) spectra of (1) Ca-alginate microparticles and (2) sodium alginate.

4.2.3.2 Ca-Yam-alginate microparticles

The FT-IR spectras of Ca-Yam-alginate microparticles are shown in Figure 39. The main peak of starches was at 537 and 930, respectively (Figure 39-1). The infrared absorption band at 930 cm^{-1} was attributed to the α-1,4glycosidic linkages in starches. The band around 1420 cm^{-1} refers to CH_2 bending, C–O–O stretch. The absorption at 1642.7 cm^{-1} is a typical band residing in the spectra of starch, which is attributed to an H_2O bending vibration (Luo et al., 2009: 563-568). Numbers of hydroxyl groups in starch molecules lead to the absorption of water. Comparing of those three graphs, the FT-IR spectrum of Ca-Yam-alginate showed the similar structure of both yam starch and Ca-alginate microparticles (Figure 39). It could be explained that yam starch was successfully incorporated into microparticles.

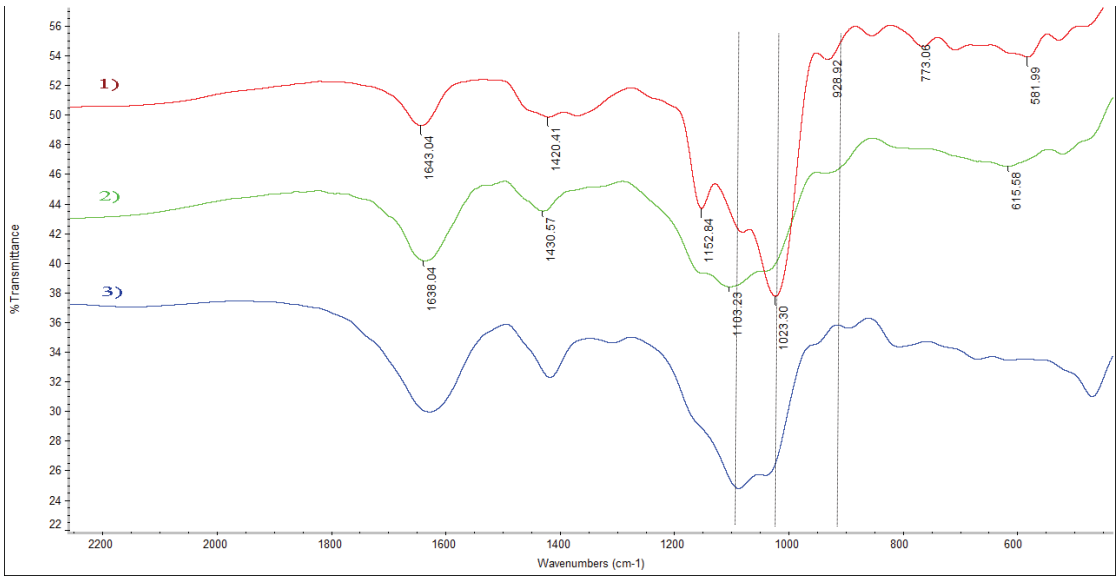

Figure 39 The FT-IR spectra of (1) yam starch, (2) Ca-alginate microparticles and (3) Ca-Yam-alginate microparticles.

4.2.3.3 CM-Ca-alginate microparticles

The chemical structures of CM-Ca-alginate microparticles were characterized by FTIR spectroscopy. Figure 40-1 depicts the characteristic FTIR pattern of CS-HCl. The absorption bands at wave numbers 3430 and 2927cm^{-1} are due to OH, CH_2 and NH_2 groups. The band of CS at about 1669 cm^{-1} (NH_2) disappears giving rise to two new bands located at 1631 and 1522 cm^{-1}. This behaviour reflects the interaction between the amino groups and the HCl, and the bands at 1155, 1081, and 1033 cm^{-1} correspond to the symmetric stretching of the C-O-C and involve the skeletal vibration of the C-O stretching (Brugnerotto et al., 2001: 3569–3580; Orienti et al., 2002: 51-59). The FT-IR spectrum of $TM_{65}CM_{50}CS$ (Figure 40-2) was similar to the spectrum of CS-HCl except that additional absorption bands at wave numbers 1611, 1518, and 801 cm^{-1} were observed. These bands were assigned to the C=C stretching and C-H deformation (out of plane) of the aromatic group. The TM-CM-CS exhibited the characteristic FT-IR spectrum at wave number 1473 cm^{-1} which was similar to the FT-IR spectrum of TM-CS and is due to C-H symmetric bending of the methyl substituent of the quaternary ammonium groups. For CM-Ca-alginate microparticles (Figure 40-3), some peaks disappeared or became weak due to

interaction or superposition between groups of alginate and chitosan. However the peak of CM-Ca-alginate microparticles still shows the same peak as a $TM_{65}CM_{50}CS$ such as peak around 1629 cm^{-1} correspond to C=C stretching of the aromatic group of $TM_{65}CM_{50}CS$ coated upon the microparticles. This result indicated that Ca-alginate microparticles were successfully coated with $TM_{65}CM_{50}CS$ on their surface.

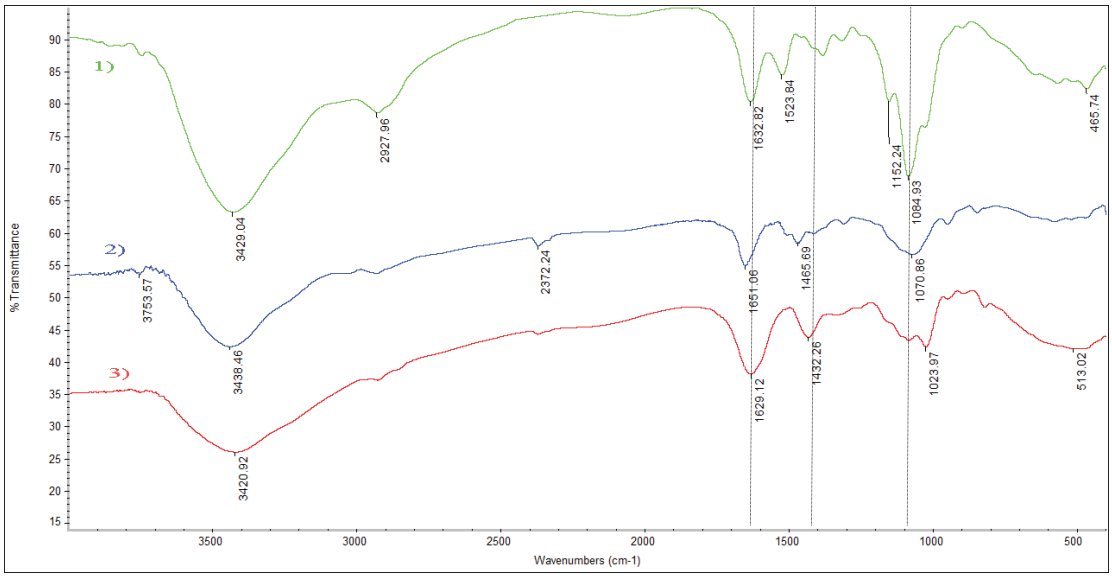

Figure 40 The FT-IR spectra of (1) CS-HCl, (2) $TM_{65}CM_{50}CS$ and (3) CM-Ca-alginate microparticles.

4.2.3.4 CM-Ca-Yam-alginate microparticles

Figure 41 shows FT-IR spectra of CM-Ca-Yam-alginate microparticles which showed the similar peak as CM-Ca-alginate microparticles. For example, the absorption bands at wave numbers 3430 and 2927 cm^{-1} are due to OH, CH_2 and NH_2 groups. Moreover, CM-Ca-Yam-alginate microparticles showed band of $TM_{65}CM_{50}CS$ at wave number 1473 cm^{-1} that is due to C-H symmetric bending of the methyl substituent of the quaternary ammonium groups and the main structure around 537 cm^{-1} and 930 cm^{-1} that refer to the α-1,4 glycosidic linkages in starches. This result indicated that CM-Ca-Yam-alginate microparticles not only had the same characteristic of Ca-Yam-microparticles but also had the characteristic of $TM_{65}CM_{50}CS$ in their structures.

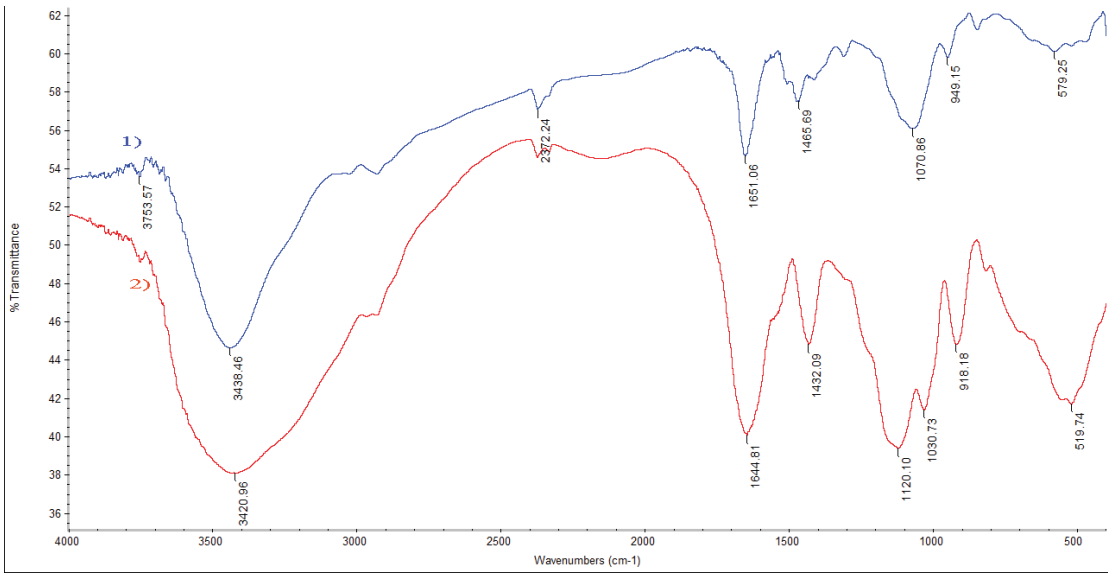

Figure 41 The FT-IR spectra of (1) $TM_{65}CM_{50}CS$ and (2) CM-Ca-Yam-alginate microparticles.

4.2.4 Swelling property

The swelling property of microparticles was conducted in phosphate buffer solution (PBS) pH 7.4. The percentage of swelling at different time interval was determined by calculating the different diameter of microparticles before and after incubation (Figure 42). As shown in Figure 42, the results revealed that all microsphere formulations swelled rapidly when immersed in phosphate buffer, especially in CM-Ca-alginate formulation. The CM-Ca-alginate formulation showed the highest percent swelling at 0.5, 1 and 2 h compared with other formulations. Increasing the time of incubation increased the percentage of swelling. It is reported that adhesive properties and cohesiveness of mucoadhesive polymers are generally affected by their swelling behavior (Mortazavi and Smart, 1993: 197–203). Mucoadhesive microspheres are anticipated to take up water from the underlying mucosal tissue by absorbing, swelling, and capillary effects, leading to considerable stronger adhesion (Duchene and Ponchel, 1992: 709–714).

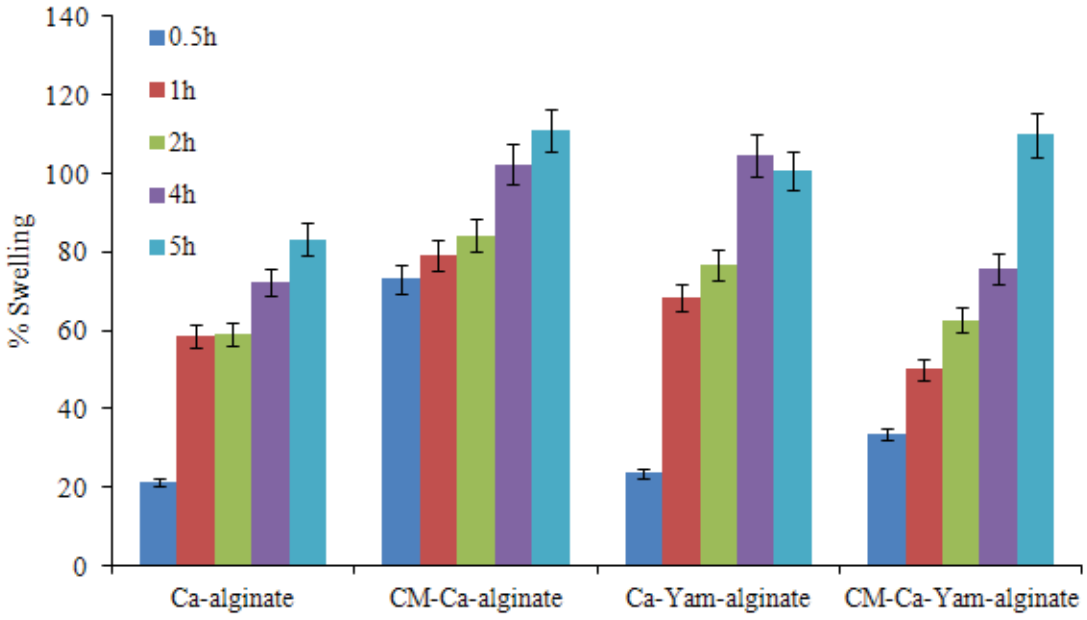

Figure 42 Percent swelling of Ca-alginate, CM-Ca-alginte, Ca-Yam-alginate and CM-Ca-Yam-alginate microsparticles at different time of incubation. The data was presented as mean ± SD (n =10).

The percent swelling of different microspheres formulation was found to follow the rank order 58.39±18%, 84.14±15%, 68.19±26% and 50±15% for Ca-alginate, CM-Ca-alginate, Ca-Yam-alginate and CM-Ca-Yam-alginate microparticles, respectively. After 2 h incubation for microspheres prepared from Ca-alginate, CM-Ca-alginate, Ca-Yam-alginate and CM-Ca-Yam-alginate and after 5 h of incubation, percent swelling was observed to be 83.21±28%, 110.97±24%, 100.70±30% and 109.89±15%, respectively. The considerably increased in the swelling of bead might be due to the swelling force resulted from the presence of counterions which neutralized created carboxylic groups on alginate at neutral media (Tavakol, 2009: 326-330). The anionic nature of alginate might enhance the repulsion among the molecular chains and resulted in an increase in swelling ratio. After adding yam starch in Ca-alginate formulation, the percent swelling was increased due to the hydrophilic nature of starch, which imparts increasing hydrophilicity to the blend and brings about an increase in swelling ratio (Roy, Bajpai and Bajpai, 2009: 222–231). In case of CM-coated microparticles, the swelling degree and swelling rate were higher than those of

uncoated microparticles. It might be resulted from the swelling of water-soluble chitosan at pH 7.4 associated of from hydrogen bonding between the amino groups within the chitosan chains (Rohindra, 2004: 32-35).

4.2.5 *In vitro* mucoadhesion study

The mucoadhesive properties of microparticles were evaluated by the everted sac experiments using small intestine of rats. Since the assay is easy to reproduce and can be done in almost any laboratory (Santos et al., 1999: 113-122; Yasunori et al., 2003: 21-29). In addition, the time course of adhering percentage was obtained by counting at appropriate intervals. The results of the experiments were presented as percentage adhering. A high percentage of adhesion indicates that microspheres have excellent mucoadhesion to mucosal tissue. Figure 43 shows the results of the everted sac experiments for the microparticles in different formulation. According to the percent adhering versus time curves, CM-Ca-alginate and CM-Ca-Yam-alginate separated rapidly, therefore the adhering percent at 1 h was 50% and 57%, respectively. Ca-alginate and Ca-Yam-alginate separated from the sacs gradually, showing an adhering percent at 1 h of 82% and 76% respectively. CM-Ca-alginate showed the highest percentage of adhesion at any time during the test, followed by CM-Ca-Yam-alginate, Ca-Yam-alginate, and Ca-alginate until 1.5 h. These results indicated that the mucoadhesive ability was affected by coating of $TM_{65}CM_{50}CS$ on the surface of microparticles. Generally, chitosan has been shown to interact with mucin (Fiebrig et al., 1994: 66–73; 1995: 239–244). Takeuchi et al., (1996: 896-900) prepared liposomes coated with chitosan and found that a prolonged residence time in the GI tract of rats in comparison to uncoated liposomes. It is reported that adhesive properties and cohesiveness of chitosan (mucoadhesive polymers) are generally affected by their swelling behavior (Mortazavi and Smart, 1993: 197–203). In the mucoadhesion process, it is necessary for swelling and expansion of the polymer chain since interpenetration and entanglement of the polymers and the mucous networks are considered to be responsible for adhesion (Duchene, Touchard and Peppas, 1988: 283–318). Therefore, bioadhesives should swell and expand rapidly when they come in contact with water. Moreover

$TM_{65}CM_{50}CS$ is a positive polymer. They have numerous hydrophilic functional groups such as carboxyl groups in chitosan molecules, which have an ability to form hydrogen bonds with the mucus molecules. This interaction is reported to be responsible for mucoadhesive property of this polymer (Harikarnpakdee, Lipipun and Sutanthavibul 2006: E2-E10). In contrast, Ca-alginate microparticles and Ca-Yam-alginate possessed a negative charge. In the presence of the PBS buffer (pH 7.4) medium, this negative charge could repelled by the negatively charged mucus, leading to poor mucoadhesion. Sumeet et al. (2008: 322-330) investigated mucoadhesive measurement of different microspheres formulation in pig intestine and reported that strong electrostatic attraction between mucin and Carbopol 71G or Chitosan seems to have contributed to good mucoadhesion. From this study, it could be indicated that $TM_{65}CM_{50}CS$ can improve bioadhesive properties of the microparticles.

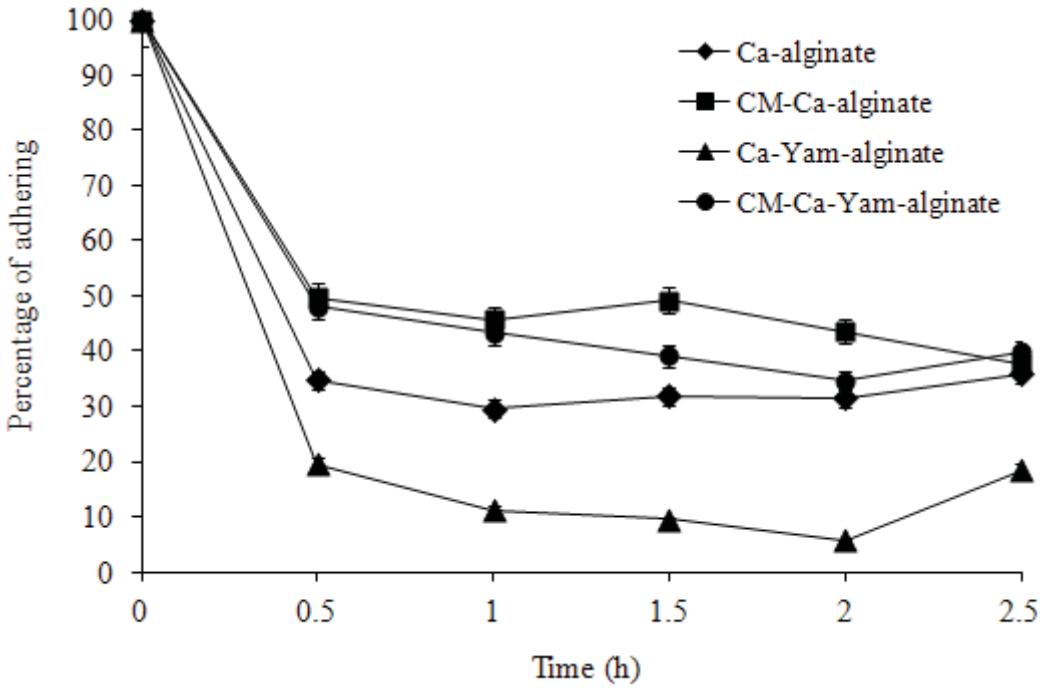

Figure 43 *In vitro* mucoadhesion of microspheres to the small intestinal of pig (◆) Ca-alginate, (■) CM-Ca-alginate, (▲) Ca-Yam-alginate, (●) CM-Ca-Yam-alginate microparticles.

4.3 Entrapment efficiency and OVA content

To ensure amount of OVA in microparticles preparation, entrapment efficiency and content of OVA should be investigated. Various amounts of initial OVA (10, 20, 40% w/w to polymer) were incorporated into the CM-Ca-Yam-alginate microparticles.

4.3.1 Ca-alginate and CM-Ca-alginate microparticles

Figure 44 shows percentage of entrapment efficiency (%EE) of (a) Ca-alginate and (2) CM-Ca-alginate microparticles. The %EE of Ca-alginate and CM-Ca-alginate microparticles was found to be in the range of 26–40% and 31–35%, respectively, depending on the initial OVA added. Ca–alginate microparticles with initial 20% w/w OVA showed the highest entrapment efficiency and amount of OVA content (40.95±0.4% and 33.22±0.1 mg/g, respectively) as similar to CM–Ca–alginate microparticles with initial 20% w/w OVA that showed the highest entrapment efficiency and amount of OVA content (35.74±0.1% and 15.43±0.5 mg/g, respectively). These results indicated that the maximum entrapment efficiency and loading capacity of OVA in Ca-alginate microparticles and CM-Ca-alginate microparticles was 20%w/w of OVA to polymer.

4.3.2 Ca-Yam-alginate and CM-Ca-Yam-alginate microparticles

In Figure 45, the entrapment efficiency of Ca-Yam-alginate and CM-Ca-Yam-alginate microparticles was found to be in the range of 25–36% and 17–25%, respectively, depending on the initial OVA added. Ca–Yam-alginate microparticles with initial 40% w/w OVA showed the highest entrapment efficiency and amount of OVA content (36.61±0.4% and 46.47±0.1 mg/g, respectively) as similar to CM–Ca-Yam–alginate microparticles with initial 40% w/w OVA that showed the highest entrapment efficiency and amount of OVA content (25.85±0.1% and 36.16±0.5 mg/g, respectively). These results indicated that the maximum entrapment efficiency and loading capacity of OVA in Ca-alginate microparticles and CM-Ca-alginate microparticles was 40%w/w of OVA to polymer.

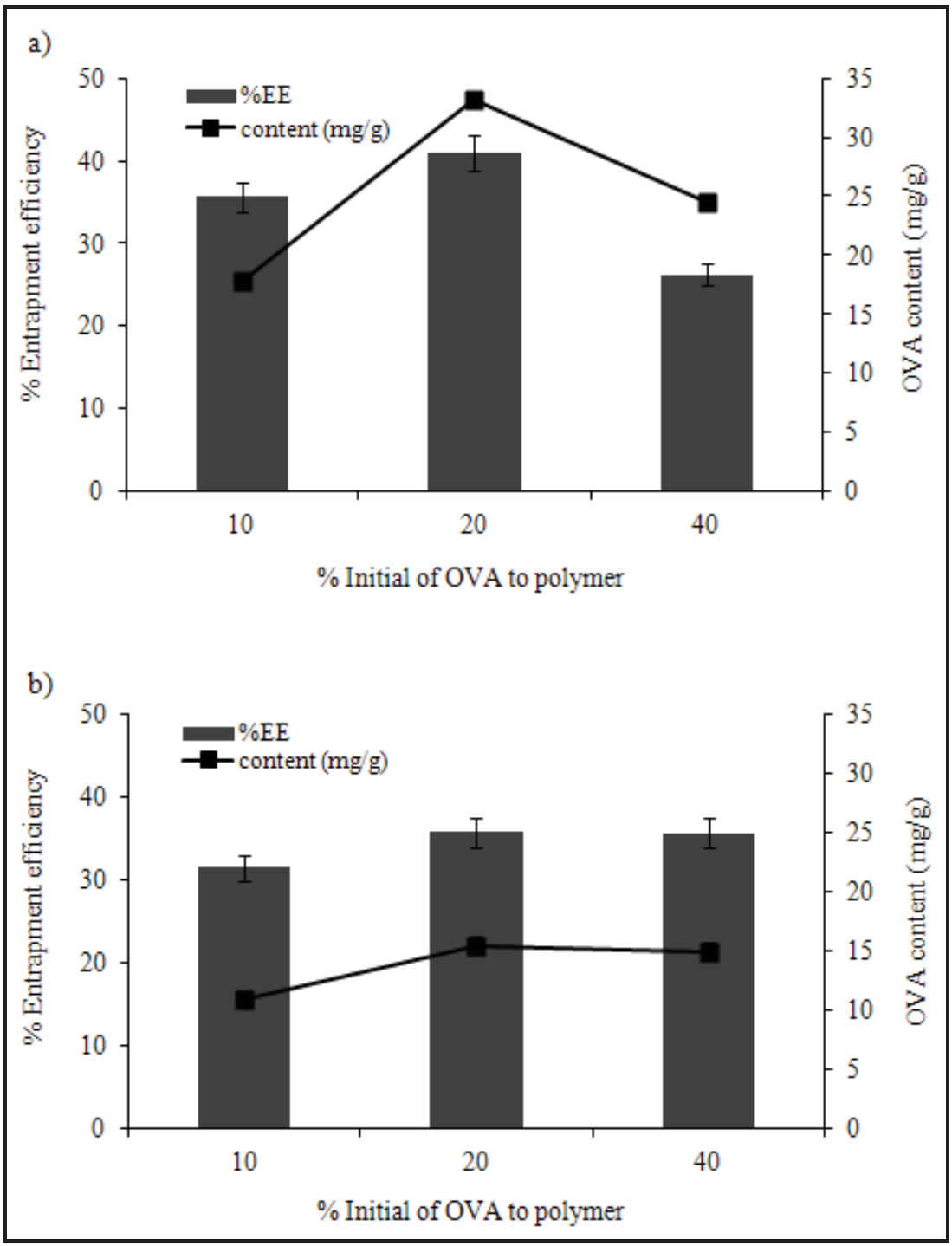

Figure 44 Percentage of entrapment efficiency and OVA content of OVA loaded (a) Ca-alginate microparticles and (b) CM-Ca-alginate microparticles.

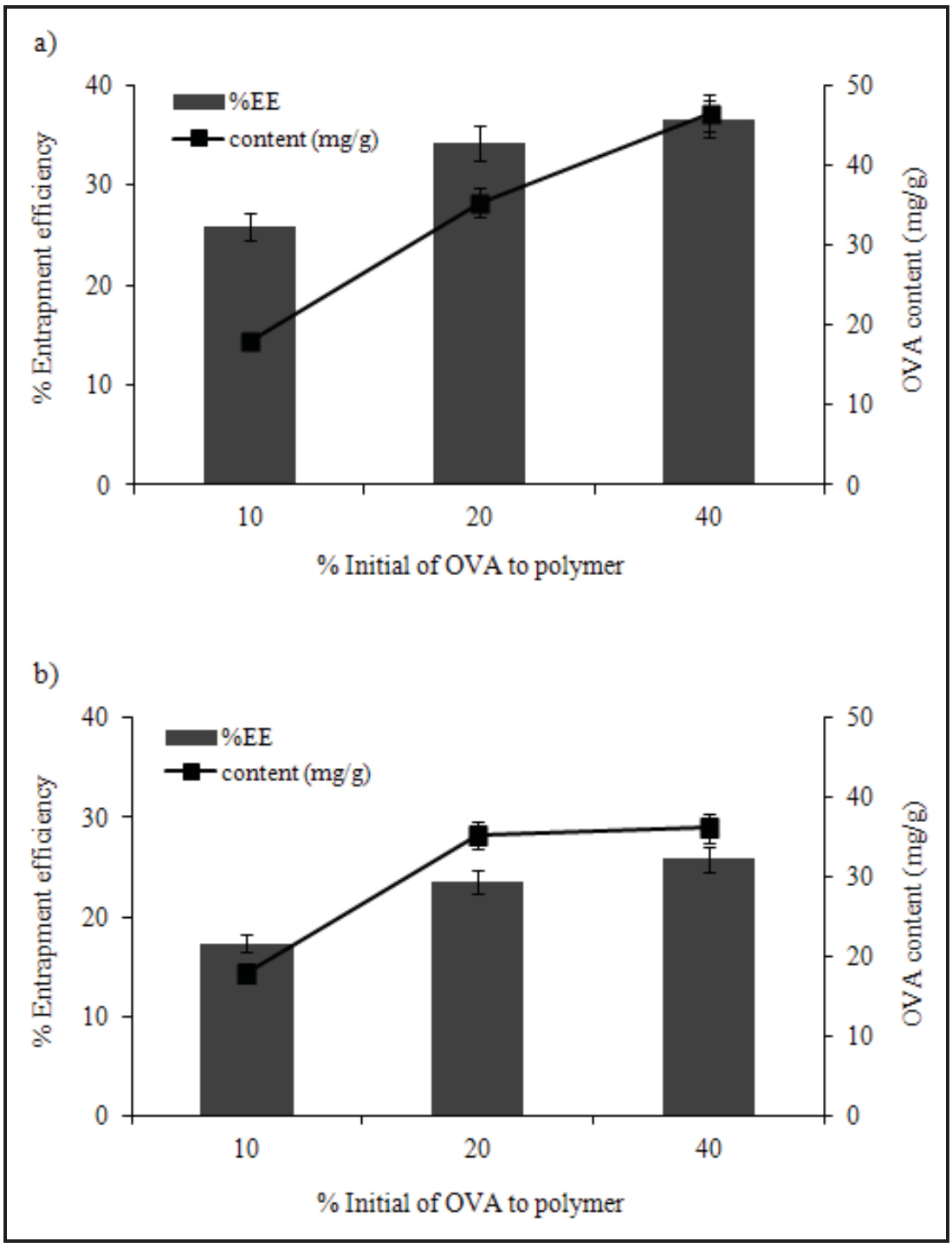

Figure 45 Percentage of entrapment efficiency and OVA content of OVA loaded (a) Ca-Yam-alginate microparticles and (b) CM-Ca-Yam-alginate microparticles.

4.4 *In vitro* release of ovalbumin (OVA) loaded microparticles

The release profile of OVA from microparticles was prolonged when coated with mucoadhesive polymers. Different types of polymer produced different drug release patterns (Figure 46 and 47).

4.4.1 Ca-alginate and CM-Ca-alginate microparticles

The release profiles of OVA from the prepared OVA-loaded Ca-alginate microparticles and CS-Ca-alginate microparticles are shown in Figure 46. The release profile of OVA from Ca-alginate microparticles was approximately 80% (Fig. 46a), whereas CM-Ca-alginate microparticles was approximately 50 % (Fig.46b) over a period of 24 h. The release rate of OVA from Ca–alginate microparticles was higher than CM–Ca–alginate microparticles, and the lowest release rate, sustained release for 24 h, was found in the initial 40% w/w OVA. These results indicated that OVA entrapped in the CM-Ca-alginate microparticles were mostly remained under these conditions. This reason could be explained that Ca-alginate microparticles have a macroporous structure and possible rapid dissolution at intestinal pH, which may cause higher release of core substances more than in the coated particles (Georg and Abraham, 2006: 1–14**).**

4.4.2 Ca-Yam-alginate and CM-Yam-Ca-alginate microparticles

The release profiles of OVA from the Ca-Yam-alginate microparticles and CM-Ca-Yam-alginate microparticles are shown in Figure 47. The result was correlated with release profiles of Ca-alginate and CM-Ca-alginate microparticles. The release profile of OVA from Ca-Yam-alginate microparticles was approximately 80% (Fig. 47a), whereas CM-Ca-alginate microparticles was approximately 30 % (Fig.47b) over a period of 24 h. The release rate of OVA from Ca–Yam-alginate microparticles was higher than CM–Ca–Yam-alginate microparticles, and the lowest release rate, sustained release for 24 h, was found in the initial 40% w/w OVA. Ca-Yam-microparticles showed higher release profile more than CM-Ca-Yam-alginate microparticles. This is due to the porous structure of yam-alginate as shown in SEM photography microparticles. Moreover, at pH 7.4, the microparticles were eroded due to the properties of alginate and native starch.

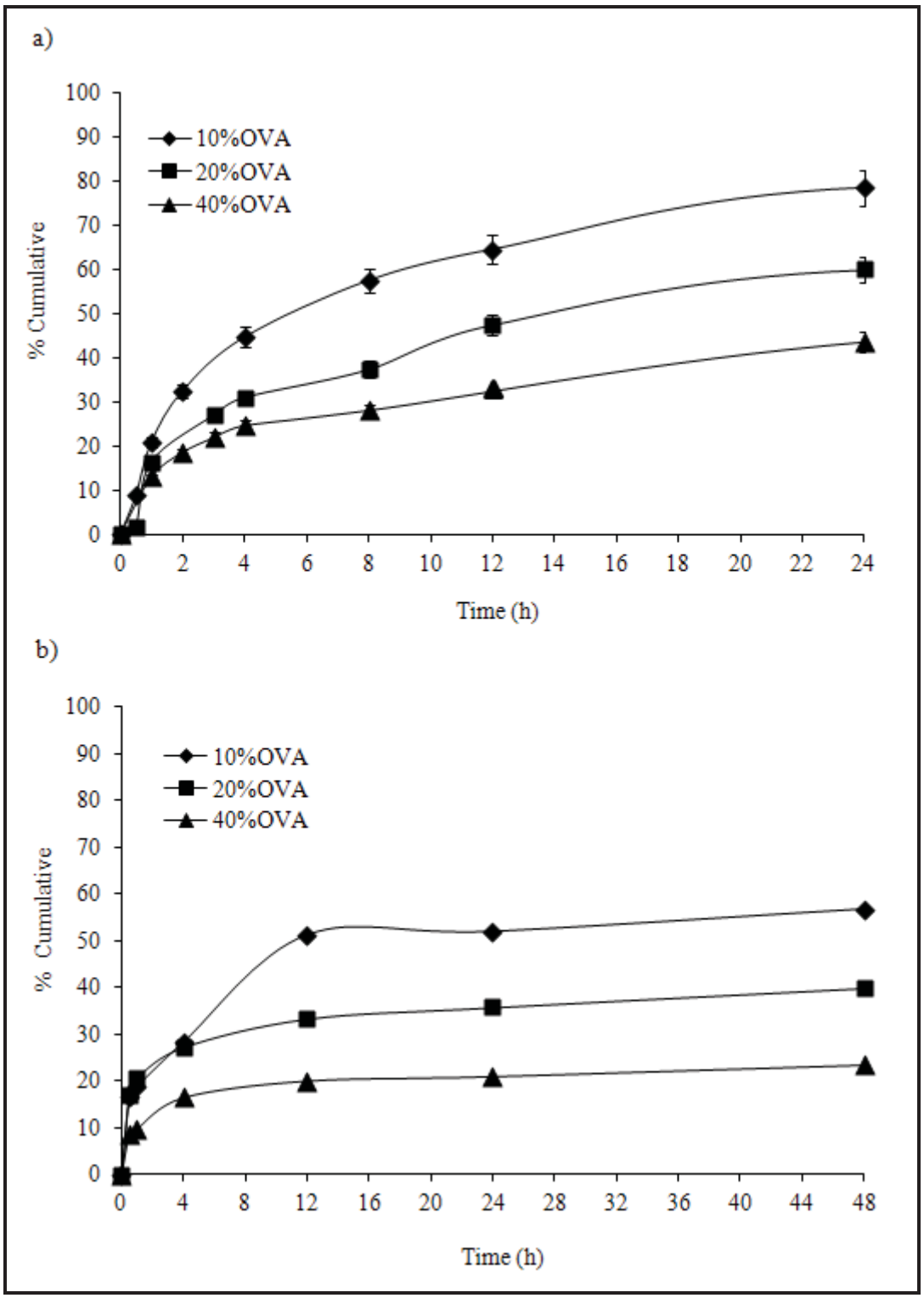

Figure 46 Release profile of OVA loaded (a) Ca-alginate microparticles and (b) CM-Ca-alginate microparticles incubation with 0.1N HCl pH1.2 for 2 h and then replaced with PBS pH 7.4 until 48 h.

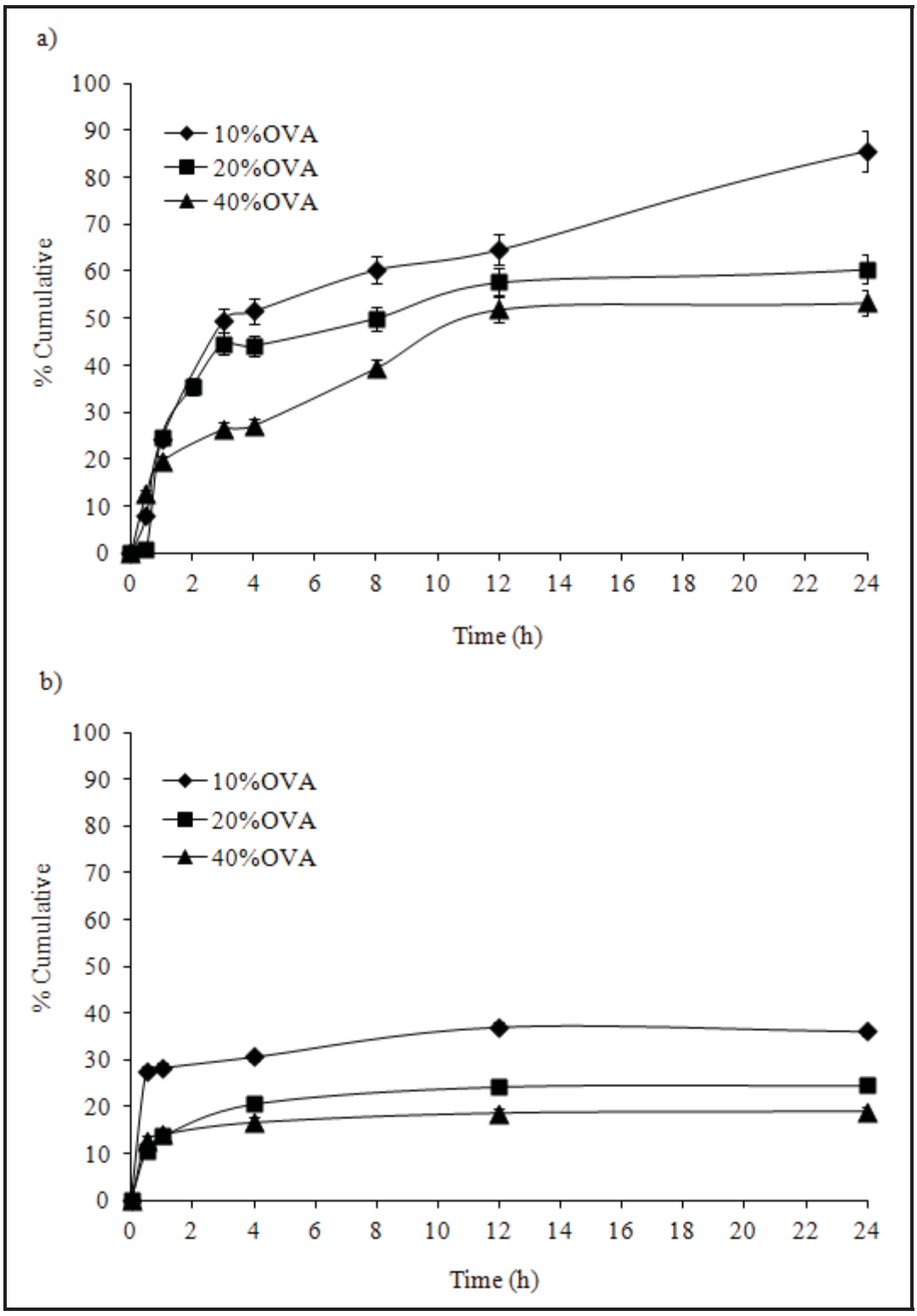

Figure 47 Release profile of OVA loaded (a) Ca-Yam-alginate microparticles and (b) CM-Ca-Yam-alginate microparticles incubation with 0.1N HCl pH1.2 for 2 h and then replaced with PBS pH 7.4 until 48 h.

All of formulations showed slow release at low pH. It is due to the limited swelling degree of alginate microparticles at low pH which resulted in decrease OVA release from microparticles. The release profile of Ca-Yam-alginate microparticles was higher than Ca-alginate microparticles. This might be due to the effect of crosslinking agent participated in release rate profile (Roy, Bajpai and Bajpai, 2009: 222–231). In Ca-Yam-alginate microparticles preparation, the concentration of $CaCl_2$ was decreased because of the addition of yam starch into structure. This process used lower crosslinking agent, resulting in higher erosion and higher release rate profiles, respectively. Moreover the effect of starch in microparticles is also participated with the release profiles. Roy, J. Bajpai and A.K. Bajpai (2009: 222–231) reported that the addition of starch, which is a non-ionic polymer brings, about weakening of intermolecular forces between the starch and alginate macromolecules and thus enhances dimensional stability of the particles. As the particles surfaces lose their integrity and the interaction between polymer chains and water molecules the percentage of erosion increased, OVA release was increased. The released of OVA from coated microparticles was relatively slower than uncoated microparticles due to the lower degree of swelling, lower rate of disintegration and resistance of the polyelectrolyte complex membrane against drug diffusion and release. The OVA release rate at pH 7.4 increased in accordance with increase in swelling degree of the hydrogel network of polymer. Tapia et al. (2004: 65-75) showed that chitosan-alginate complex erodes slowly in phosphate buffer at pH values higher than 6.5 and this behavior leads to suppression of the initial drug release in the upper segments of GIT occurring for uncoated micro-particles and controls release in the colon.

4.5 Cytotoxicity of microparticles

Although IC_{50} of $TM_{65}CM_{50}CS$ solution was known, the cytotoxicity of new formulation must be investigated. The cytotoxicity effects of $TM_{65}CM_{50}CS$ coated and uncoated microparticles on Caco-2 cells were determined as % cell viability as shown in Figure 48. The concentration-dependent cytotoxicity of microparticles was observed, when incubated for 24 h. IC_{50} value, indicating 50% cell deactivation, of all formulation was more than 20 mg/ml, indicating that no toxic occurred after incubation with microparticles over 24 h. It could be indicated that all

formulation of microparticles prepared in this study were safe. It is due to the nature of alginate and starch, which are natural polymer. The findings correlated with results of other studies which indicate that both alginate and starch microparticles showed a good indication of their noncytotoxic behavior (Suksumran et al., 2011: 317-324; Silva et al., 2004: 442-449). $TM_{65}CM_{50}CS$ solution showed a slightly toxicity (section 4.1.5.2), however when $TM_{65}CM_{50}CS$ was coated with microparticles, there was no cytotoxicity at the same concentrations (Figure 48). From this result, it was assured that the coated and uncoated particles were safe in the *in vitro* cytotoxicity.

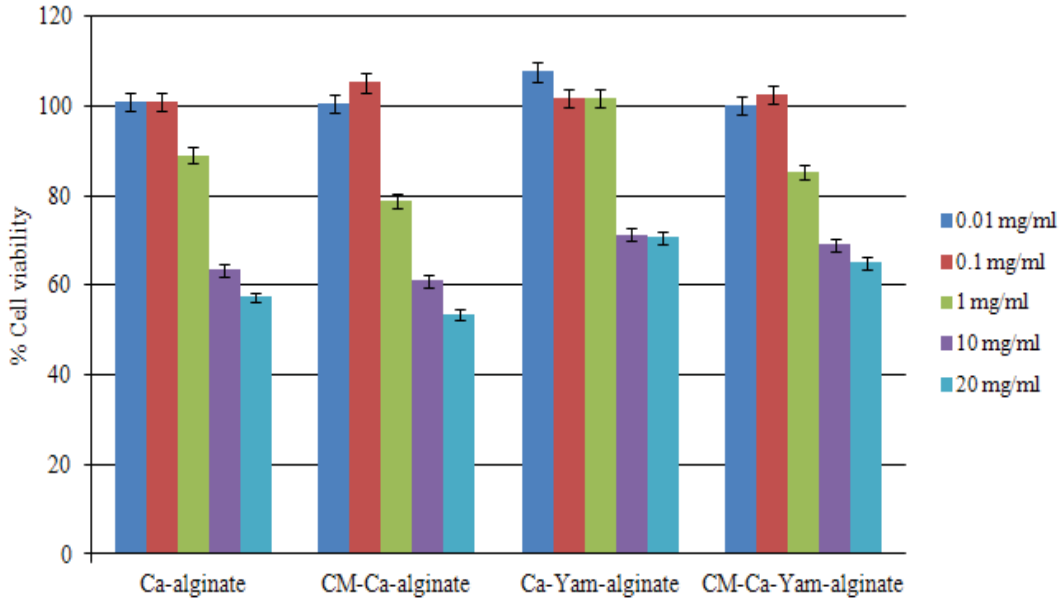

Figure 48 % Cell viability of coated and uncoated microparticles incubated with Caco-2 cells for 24 h (n=8)

4.6 *In vivo* immunological study

4.6.1 OVA-specific immunoglobulin G (IgG) antibody

BALB/c mice were intragastrically administered 500 µl of PBS pH 7.4 solution containing OVA-entrapped microparticles (20 mg eq. to OVA 250 µg). Control animals were received free OVA (250 ug in PBS pH 7.4) via the same route. Serum from the mice was collected before the first immunization and after the boost.

The serum was analyzed by ELISA as previously described (Boonyo et al., 2007: 168–175). With microparticles produced from Ca-alginate, CM-Ca-alginate, Ca-Yam-alginate and CM-Ca-Yam-alginate, serum IgG antibody responses to entrapped OVA were observed (Figure 49).

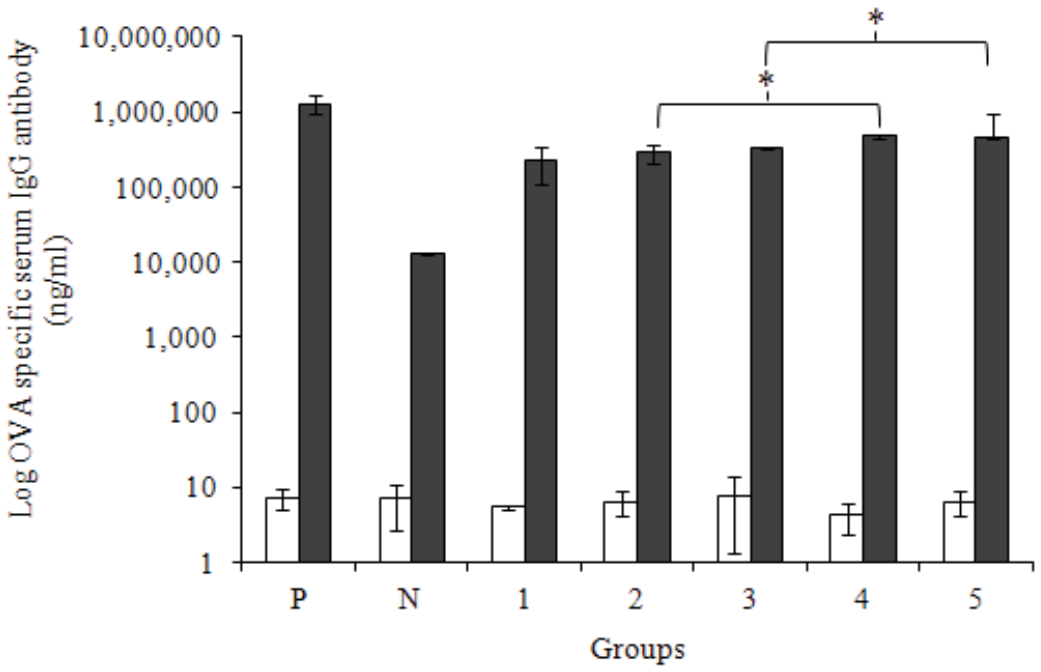

Figure 49 Serum IgG antibody titers obtained from mice following oral immunization with OVA in either PBS pH 7.4 solution (group N), OVA in 0.1 %w/v of $TM_{65}CM_{50}CS$ solution (group 1), OVA loaded microparticles; Ca-alginate (group 2), Ca-Yam-alginate (group 3), CM-Ca-alginate (group 4), CM-Ca-Yam-alginate (group 5). OVA in alum (group P) administered subcutaneously was used as a positive control. White bars show IgG titers at day 0 while black bars show IgG titers at day 21. (*) indicates $p<0.05$.

The results showed that all mice vaccinated with OVA loaded microparticles produced a strong and significant enhancement in IgG titres against OVA. Only small immune responses were present in the groups vaccinated with OVA in PBS. The reason why mice those received OVA in PBS solution yield low in immune response was due to the adverse environment of the gastrointestinal tract, rich in acids and enzymes, which are able to destroy the antigen. From this reason,

OVA loaded microparticles in all formulation yielded higher IgG titres more than mice those received OVA alone in solution. Comparing the results of microparticles, IgG levels of mice those received OVA loaded coated micropartcles were significantly higher than those received OVA loaded uncoated microparticles. This might be the effect of $TM_{65}CM_{50}CS$ that coated on microparticles. $TM_{65}CM_{50}CS$ is a positive polymer that coated on the surface of microparticles. The charge of polymer made coated microparticles also yielded in positively charge (Table 11) and associated with bioadhesive properties (Figure 43). When mice received OVA loaded microparticles, these positive charges of coated microparticles contributed to the mucoadhesive property of chitosan by forming strong electrostatic interaction with the negatively charged sialic acid residues of mucins, thus resulting in increase of half time of clearance of an antigen (Illum et al., 2001: 81-96; Soane et al., 1999: 55-65). The result was correlated with the result of bioadhesive properties of the microparticles that coated microparticles showed higher bioadhesive properties than those uncoated microparticles.

4.6.2 OVA-specific immunoglobulin A (IgA) antibody

IgA and IgG responses of the various groups of mice were similar (Figure 50). There was very low IgA response observed when mice were immunized s.c. with OVA in alum (group P). The IgA levels obtained from the mice those received OVA in coated microparticles were higher than OVA in uncoated microparticles. The level of the IgA response obtained from mice immunized with OVA in CM-Ca-alginate was slightly higher than OVA in CM-Ca-Yam-alginate microparticles. This result could be concluded that oral vaccine delivery can induce both systemic and mucosal immune response. Moreover, the mice those received entrapped OVA within microparticles showed higher IgA responses than mice those received OVA solution (group N and 1). It could be explained that association of the vaccine with microparticulate drug carrier systems may prevent its degradation in the stomach and the gut and may stimulate the M-cells to transport the vaccine to the dome of the Peyer's Patchs. After transportation of the microparticles to the dome of the PPs, the microparticles are degraded and the vaccine is released into the lymphoid tissue,

resulting in the immune response (Alpar, Ward and Williamson, 2000: 269–278; Lubben et al., 2001; 39–47; Barackman et al., 1998: 41–46).

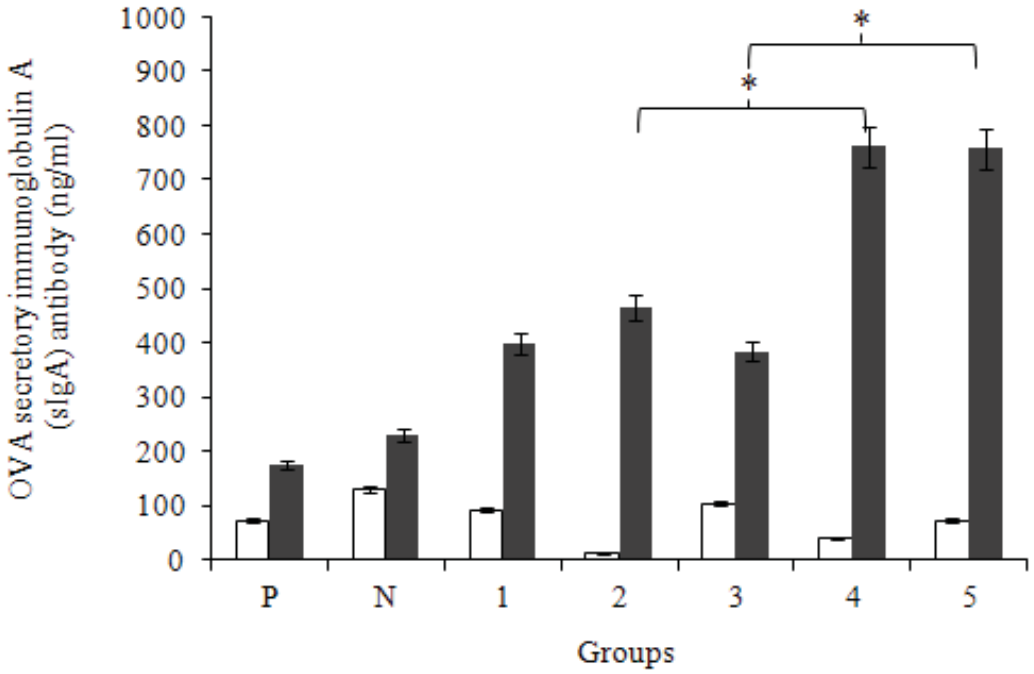

Figure 50 IgA antibody titers obtained from mice following oral immunization with OVA in either PBS pH 7.4 solution (group N), OVA in 0.1 %w/v of $TM_{65}CM_{50}CS$ solution (group 1), OVA loaded; Ca-alginate (group 2), Ca-Yam-alginate (group 3), CM-Ca-alginate (group 4), and CM-Ca-Yam-alginate (group 5). OVA in alum (group P) administered subcutaneously was used as a positive control. White bars show IgA titers at day 0 while black bars show IgA titers at day 21. (*) indicates $p<0.05$.

CHAPTER 5
CONCLUSIONS

This work demonstrates the effects of microparticles formulation and process variables on particle size, entrapment efficiency, *in vitro* release, mucoadhesiveness and especially on the *in vivo* immunogenicity of microparticles which made from alginate or yam-alginate as a core and chitosan derivatives as the outer coating. In this study microparticles from calcium-alginate (Ca-alginate), $TM_{65}CM_{50}CS$ coated calcium-alginate (CM-Ca-alginate), calcium-yam-alginate (Ca-Yam-alginate) and $TM_{65}CM_{50}CS$ coated calcium-yam-alginate (CM-Ca-Yam-alginate) were successfully prepared by ionotropic gelation method using electrohydrodynamic atomization (EHDA) technique. The appropriate condition for preparation of Ca-alginate microparticles was 1%w/v alginate (low viscosity) + 4%w/v $CaCl_2$ and 1%w/v alginate (low viscosity) + 1%w/v Yam starch + 4%w/v $CaCl_2$ for Ca-Yam-alginate microparticles. These formulations were chosen to be incorporated with ovalbumin as a model antigen. The size of microparticles was in range 1-3 µm and the shape was spherical.

Chitosan derivatives including $TM_{65}CS$, $TM_{65}CM_{50}CS$, $TM_{56}Bz_{42}CS$ and $TM_{53}Py_{40}CS$ were investigated for their cytotoxicity and immunological activity. Among these chitosan derivatives, $TM_{65}CM_{50}CS$ showed the highest immunological activity by inducing the highest systemic immune response (IgG) and mucosal immune response (IgA) titre. Therefore, the $TM_{65}CM_{50}CS$ was chosen for coating the microparticles. The optimized concentration of $TM_{65}CM_{50}CS$ was 0.1 %w/v. After coating the microparticles with 0.1 %w/v of $TM_{65}CM_{50}CS$, the zeta potential of microparticles was changed from negative to positive charge. The morphology of coated microparticles was spherical shape with smooth surface.

All formulations rapidly swelled after 0.5 h. CM-Ca-alginate microparticles yielded the highest swelling properties compared with other

formulations. The swelling properties of coated microparticles were higher than uncoated microparticles and correlated well with mucoadhesive properties.

The result of OVA content and entrapment efficiency of microparticles revealed that at initial 40% OVA yielded the highest entrapment efficiency and OVA content. The entrapment efficiency and OVA content of coated microparticles were lower than uncoated microparticles. The entrapment efficiency of Ca-alginate, Ca-Yam-alginate, CM-Ca-alginate and CM-Ca-Yam-alginate microparticles with initial 40% OVA to polymer were 26.27±0.4%, 36.61±0.8, 35.66±0.7 and 25.85±0.5 respectively. The content of Ca-alginate, Ca-Yam-alginate, CM-Ca-alginate and CM-Ca-Yam-alginate microparticles with initial 40% OVA to polymer were 24.49±0.4%, 46.47±0.9, 14.95±0.6 and 36.16±0.8, respectively.

The *in vitro* release of microparticles showed that the release profile of Ca-alginate (80% within 24 h) and Ca-Yam-alginate was higher (90% within 24 h) than coated CM-Ca-alginate (60% within 24 h) and CM-Ca-Yam-alginate (40% within 24 h). Ca-alginate and Ca-Yam-alginate showed burst release effect at initial time whereas coated microparticles did not. This study could be concluded that coated microparticles resulted in more sustained release than uncoated microparticles.

Cytotoxicity of all formulations showed that the IC_{50} values of all microparticles were more than 20 mg/ml which indicated a low cytotoxic. It could be concluded that the prepared microparticles were safe.

In *vivo* immunological study**,** IgG titres was very high in these mice received OVA in alum (group P). While mice those received OVA solution alone (group N) showed a very low IgG. In IgA results, there was very low in IgA response observed when mice were immunized s.c. with OVA in alum (group P). The rank of IgG and IgA titres of OVA loaded microparticles were CM-Ca-alginate, CM-Ca-Yam-alginate, Ca-Yam-alginate and Ca-alginate microparticles, respectively.

From these study, it could be concluded that $TM_{65}CM_{50}CS$ coated microparticles represented a useful carrier from their mucoadhesive properties, sustained release, and immunogenicity improvement in oral availability of model antigen. Therefore, $TM_{65}CM_{50}CS$ coated microparticles might be used as potential polymeric carrier for oral vaccine delivery.

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APPENDIX

APPENDIX A

Standard curve

1. Determination the amount of BSA

Standard : BSA

Method : Bradford assay

Detector : The wavelength at 550 nm

Concentration (µg/ml) : 12.5, 25, 50, 100, 200

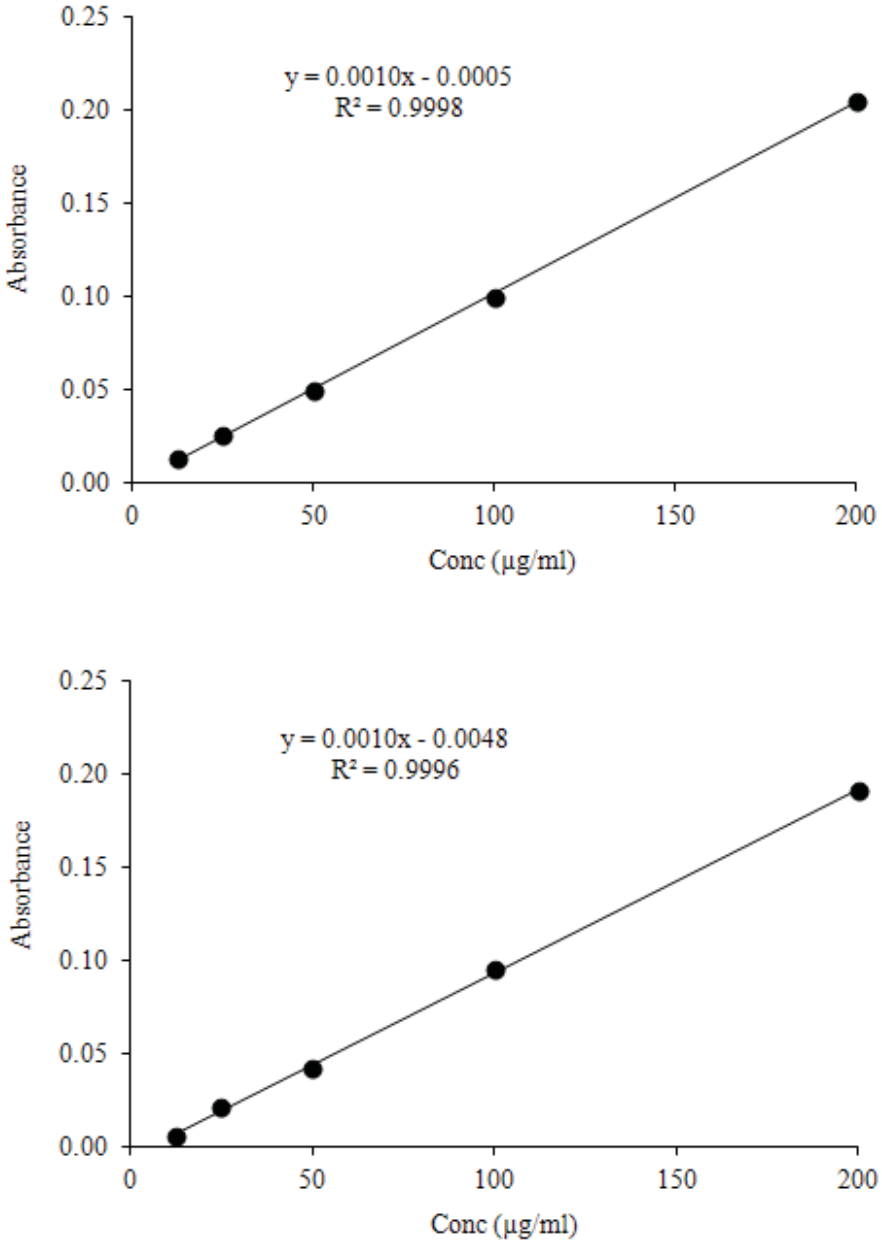

Figure 51 Standard curve of BSA

2. Determination the amount of OVA

Standard	:	OVA
Method	:	Lowry assay
Detector	:	The wavelength at 550 nm
Concentration (µg/ml)	:	12.5, 25, 50, 100, 200

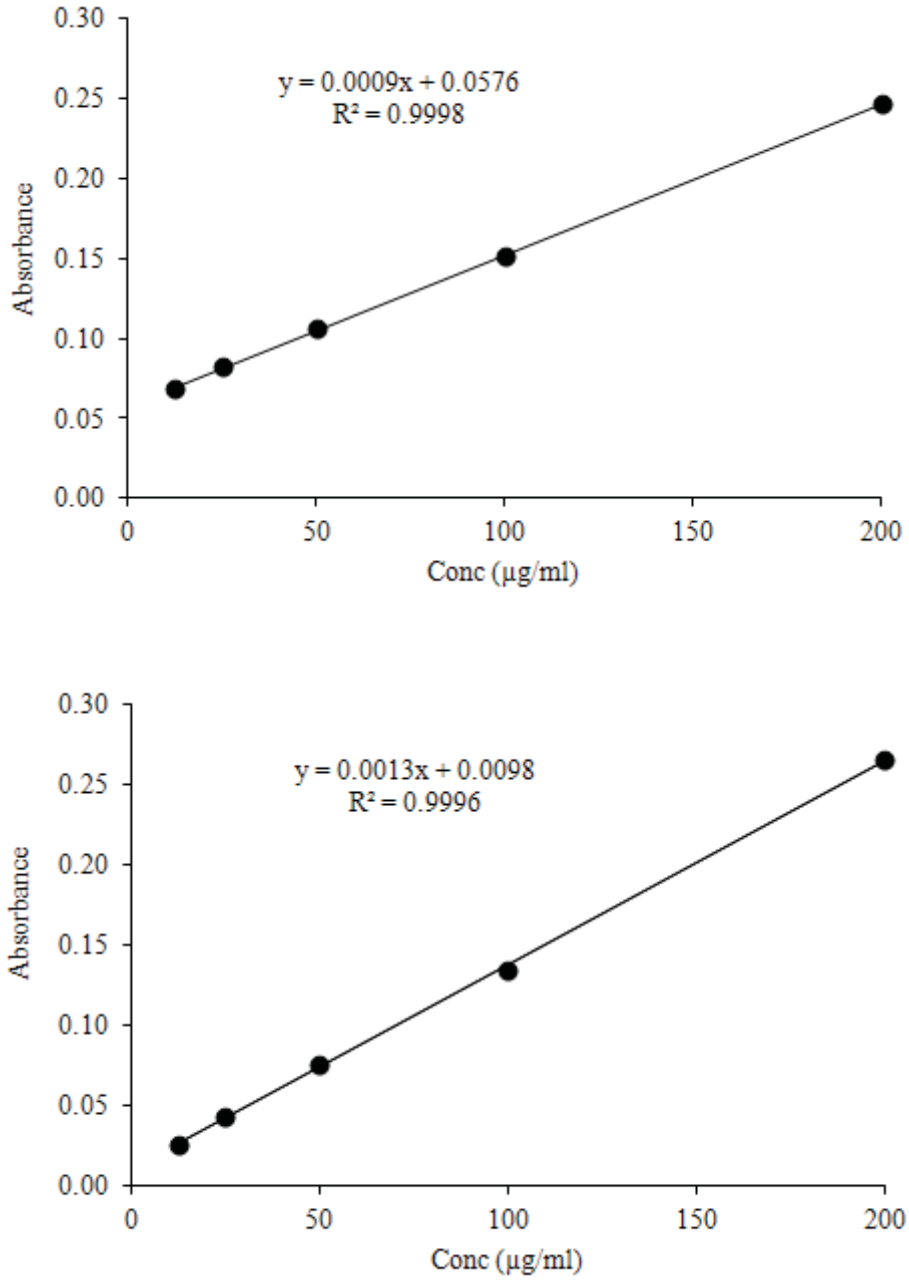

Figure 52 Standard curve of OVA

APPENDIX B

In vitro protein release

Table 12 %Cumulative of OVA with various initial OVA from Ca-alginate microparticles

Time (h)	% Cumulative of OVA					
	10%OVA		20%OVA		40%OVA	
	Ave	SD	Ave	SD	Ave	SD
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	8.80	1.38	1.59	1.99	12.70	1.66
1	20.87	5.71	16.45	1.69	13.02	2.13
2	32.45	4.11	20.15	7.52	18.61	0.11
3	38.20	1.27	27.00	1.17	21.95	3.97
4	44.65	5.02	31.01	3.15	24.71	3.84
8	57.46	9.11	37.33	3.46	28.12	5.32
12	64.51	13.66	47.46	1.98	35.33	6.19
24	78.45	10.40	60.00	8.23	43.63	3.78
48	78.51	13.20	60.33	5.76	52.70	1.42

Table 13 %Cumulative of OVA with various initial OVA from Ca-Yam-alginate microparticles

Time (h)	% Cumulative of OVA					
	10%OVA		20%OVA		40%OVA	
	Ave	SD	Ave	SD	Ave	SD
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	7.88	5.09	0.64	1.96	12.68	1.24
1	24.43	1.38	24.79	1.18	19.53	0.29
2	32.75	0.54	25.72	2.40	24.69	1.50
3	49.45	1.54	35.51	3.80	26.39	3.89
4	51.53	7.34	44.63	2.23	27.23	2.16
8	60.36	9.61	44.24	7.78	39.43	1.85
12	64.62	6.41	50.04	4.70	51.91	0.01
24	85.65	5.64	57.68	1.60	53.35	3.16
48	115.09	6.77	60.50	2.46	58.85	5.03

Table 14 %Cumulative of OVA with various initial OVA from CM-Ca-alginate microparticles

Time (h)	% Cumulative of OVA					
	10%OVA		20%OVA		40%OVA	
	Ave	SD	Ave	SD	Ave	SD
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	16.49	1.82	17.15	4.04	8.72	0.51
1	18.73	1.05	20.67	2.96	9.80	1.64
2	21.57	2.65	20.19	0.13	11.30	0.65
3	24.07	2.10	21.66	0.91	11.49	0.55
4	28.23	0.31	27.19	1.91	16.48	0.50
8	44.42	3.13	29.20	0.74	18.10	1.96
12	51.11	0.72	33.29	0.82	20.08	1.01
24	51.84	7.25	35.76	0.13	21.03	0.63
48	56.62	4.19	39.88	1.19	23.50	0.43

Table 15 %Cumulative of OVA with various initial OVA from CM-Ca-Yam-alginate microparticles

Time (h)	% Cumulative of OVA					
	10%OVA		20%OVA		40%OVA	
	Ave	SD	Ave	SD	Ave	SD
0	0.00	0.00	0.00	0.00	0.00	0.00
0.5	27.67	1.33	10.67	0.02	12.64	0.04
1	28.39	0.44	13.65	0.30	14.07	0.19
2	28.41	0.39	17.14	0.53	14.35	0.23
3	31.83	0.97	19.86	0.18	14.55	0.19
4	30.72	1.15	20.62	0.11	16.61	0.36
8	32.66	1.85	21.82	1.90	16.69	0.59
12	37.12	1.26	24.28	0.07	18.63	0.44
24	36.16	1.95	24.56	0.39	18.99	0.64
48	42.97	1.65	29.03	0.65	19.79	0.76

APPENDIX C

Evaluation of cytotoxicity

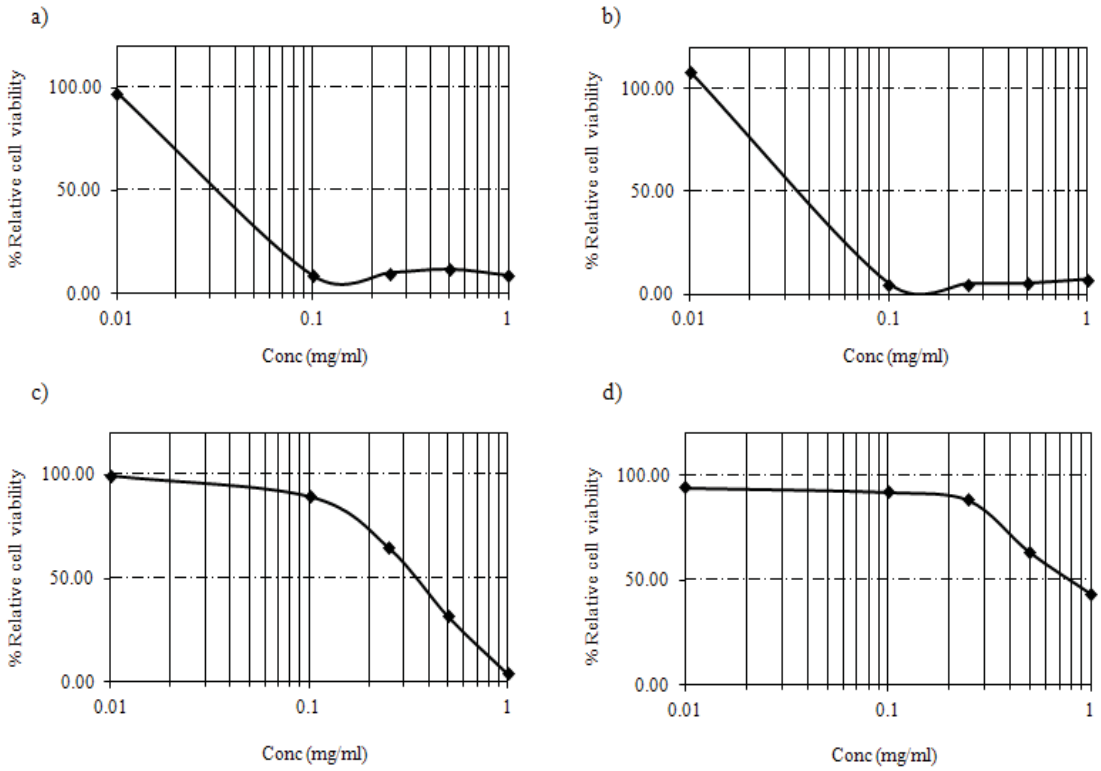

Figure 53 Cytotoxic effect of CS derivatives; a) $TM_{65}CS$, b) $TM_{65}CM_{50}CS$, c) $TM_{56}Bz_{42}CS$, and d) $TM_{53}Py_{40}CS$ concentrations from 0.01-1 mg/mL incubation for 24 h on Caco-2 cell monolayers at pH 7.4 (n=8).

APPENDIX D

LIST OF ABBREVIATIONS

%EE	Percent entrapment efficiency
%w/v	Percent weight by volume
%w/w	Percent weight by weight
µ	Microliter
°C	Degree celsius
µg	Microgram
µm	Micrometer
^{1}H-NMR	Proton nuclear magnetic resonance
Caco-2 cells	Human colon adenocarcinoma cell lines
Conc.	Concentration
CS	Chitosan
DMSO	Dimethyl sulfoxide
DQ	Degree of quaternization
ES	Extent of hydrophobic substitution
et al.	and others
FT-IR	Fourier transform infrared
g	Gram(s)
GlcN	Glucosamine
GlcNAc	*N*-acetyl glucosamine
h	Hour(s)
HPLC	High Performance Liquid Chromatography
IC_{50}	The half maximal inhibitory concentration
kDa	Kilodalton
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW	Molecular weight

PBS	Phosphate buffer solution
PDI	Polydispersity index
pH	Potentia hydrogenii (lat.)
ppm	Parts per million
R^2	Coefficient of determination
SD	Standard deviation
TM-Bz-CS	Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan
TM-CM-CS	Methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan
TM-CS	*N*,*N*,*N*-Trimethylammonium chitosan
TM-Py-CS	Methylated *N*-(4-pyridylmethyl) chitosan
UV	Ultraviolet

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Presentation

Poster :

Tittaya Suksamran, Praneet Opanasopit, Theerasak Rojanarata, Tanasait Ngawhirunpat, Uracha Ruktanonchai, Pitt Supaphol (2008), "Development of protein delivery system using alginate micro/nanoparticles" 25th Annual Research Conference in Pharmaceutical Sciences at Chulalongkorn University.

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Oral :

Tittaya Suksamran, Praneet Opanasopit, Theerasak Rojanarata, Tanasait Ngawhirunpat, Uracha Ruktanonchai, Pitt Supaphol (2008) "Development of protein delivery system using alginate micro/nanoparticles" The 2nd Thailand Nanotechnology Conference Nanomaterials for Health, Energy, and Environment, 13-15 August 2008, Phuket, Thailand.

Tittaya Suksamran, Jariya Kowapradit, Tanasait Ngawhirunpat, Theerasak Rojanarata, Warayuth Sajomsang, Tasana Pitaksuteepong, Praneet Opanasopit (2012) "Methylated *N*-aryl chitosan derivatives for oral inducing immune responses to ovalbumin" The 2nd Current Drug Development International Conference, 2-4 May 2012, Phuket, Grace Land Resort & Spa, Phuket, Thailand.

Scientific Publication

Praneet Opanasopit, Uracha Ruktanonchai, Orawan Suwantong, Suwannee Panomsuk, Tanasait Ngawhirunpat, Chavalit Sittisombut, Tittaya Suksamran, Pitt Supaphol. "Electrospun polyvinyl alcohol polymeric fibrous system as carriers for extracts from fruit hull of mangosteen", *International Journal of Cosmetic Science*, 2008; 59(3): 233-42.

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Orapan Paecharoenchai, Tittaya Suksamran, Theerasak Rojanarata, Tanasait Ngawhirunpat and Praneet Opanasopit, "Chitosan Coated Aginate Microparticles For Oral Vaccine Drug Delivery", *Advanced Materials Research*, 2011; 194-196: 541-544.

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